Gene Expression, Vol. 5, pp. 123–132, 1995
Printed in the USA.

1052-2166/95 $5.00 + .00

Functional Expression and Peroxisomal Targeting of Rat Urate Oxidase in Monkey Kidney Cells

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Humans and hominoid primates lack the enzyme urate oxidase, which catalyzes the oxidation of uric acid to allantoin. In rats and most other mammals, urate oxidase is present as a crystalloid core within the peroxisomes of liver parenchymal cells. To determine whether functionally active recombinantly expressed urate oxidase can be targeted to the peroxisome as well as display the crystalloid core-like structure, we expressed rat urate oxidase cDNA in African green monkey kidney cells (CV-1 cells) under the control of a cytomegalovirus promoter. Cell lines stably expressing urate oxidase were isolated. Northern blot analysis revealed a 1.3-kb transcript and immunoblot analysis confirmed the presence of urate oxidase in the stably transfected cells. The recombinant urate oxidase expressed in CV-1 cells was functionally active. Immunofluorescence microscopy revealed that the expressed protein was visualized as discrete granules in the cytoplasm. Electron microscopy and immunocytochemical localization studies showed that the recombinantly expressed protein formed distinct crystalloid core structures with bundles of tubules within single membrane limited cytoplasmic organelles. On cross section, the recombinant urate oxidase tubular structures are arranged as circles of 10 surrounding a slightly larger circle. This arrangement is reminiscent of urate oxidase-containing cores in rat liver peroxisomes. Immunocytochemical studies confirmed that the recombinantly expressed urate oxidase is correctly targeted to the catalase-containing peroxisomes in these CV-1 cells.

Urate oxidase Monkey kidney cells Peroxisomal targeting Functional expression

URIC acid, a product of purine catabolism, is degraded via oxidation to allantoin by the action of urate oxidase (14). Hydrolysis of allantoin by allantoinase results in the formation of allantoic acid, which is further metabolized by allantoicase to yield urea and glyoxylic acid. The enzyme urease then degrades urea to produce ammonia and carbon dioxide. In the course of phylogenetic evolution, the chain of enzymes necessary to convert uric acid to its metabolic products has been progressively truncated through the successive loss of urease, allantoicase, allantoinase, and urate oxidase (15). Most mammals, with the exception of human and hominoid primates, have urate oxidase activity in their livers and excrete allantoin as the end product of purine metabolism because these animals have lost allantoicase and allantoinase (14). The absence of urate oxidase activity in humans and hominoid primates accounts for the urinary excretion of uric acid in these species, whereas the presence of all the enzymes responsible for uric acid metabolism in lower vertebrates (e.g., fish and frog) results in the breakdown of uric acid all the way to ammonia and carbon dioxide. The relatively higher level of uric acid in humans and hominoid primates is postulated to contribute to the increased life span and decreased incidences of cancer compared to other verte-

Received July 7, 1995; revision accepted August 3, 1995.
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brates, in view of the potent antioxidant properties exhibited by uric acid (3). We and others have shown that the loss of urate oxidase activity in humans and hominoid primates, such as chimpanzee, gorilla, and orangutan, is due to the presence of nonsense mutations in the urate oxidase gene, and as a result they excrete uric acid as the end product of purine metabolism (28–31).

In most mammals that display urate oxidase activity, the enzyme appears as crystalloid cores localized within the peroxisomes of the liver (24). In amphibians and certain bovine and ovine species, the enzyme is present within the peroxisomes of liver and kidney (4,24,25,31). Urate oxidase is relatively insoluble at neutral pH and is resistant to solublization by various treatments, probably because of its crystalloid-like structure. Recombinant urate oxidase overexpressed in *Spodoptera frugiperda* (sf9) insect cells appeared randomly in both the nucleus and cytoplasm of infected insect cells (2). Because no distinct peroxisomes were identifiable in these insect cells, targeting of recombinant urate oxidase to peroxisomes could not be ascertained. To determine if the recombinant protein can be targeted to the peroxisomes in a primate cell line that lacks measurable urate oxidase activity and also shows no crystalloid core in peroxisomes, we expressed rat urate oxidase in African green monkey kidney cells (CV-1 cells). We report the expression of functionally active recombinant urate oxidase that is targeted to the peroxisomes and that the recombinantly expressed protein forms the crystalloid core-like structures.

MATERIALS AND METHODS

Plasmid Construction

The full-length rat urate oxidase (UOX) cDNA (1,21) was ligated into the Pvu II site of hygromycin-resistant pCEP4 mammalian expression vector (Invitrogen) in the sense orientation. The cDNA was under the control of human cytomegalovirus (CMV) immediate early gene enhancer/promoter. The vector contains polyadenylation signal and transcription termination sequences from SV40 and a hygromycin-resistant gene driven by thymidine kinase (TK) promoter as a selection marker. The resulting plasmid was designated pCEP-UOX (Fig. 1A).

Cell Culture and Transfection

African green monkey kidney cells (CV-1 cells), which have no urate oxidase activity and no crystalloid cores within their peroxisomes, were grown in minimal essential medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units of penicillin per ml, and 100 μg of streptomycin per ml, at 37°C in a humidified incubator with 5% CO_2/95% air atmosphere. These cells were plated at 5×10^5 cells per dish on 100-mm tissue culture dishes and transfected with 10 μg of pCEP-UOX DNA by the standard calcium phosphate precipitation method (5). After incubation for 16 h with the DNA, the dishes were washed with phosphate-buffered saline (PBS) (150 mM NaCl/10 mM sodium/potassium phosphate, pH 7.2) and reincubated with fresh medium containing 200 μg of hygromycin per ml. The medium was replaced once every 4 days and individual hygromycin-resistant colonies were obtained by the ring isolation method and subcultured separately.

Northern and Immunoblot Analyses

Total cellular RNA was prepared from untransfected CV-1 cells and the hygromycin-resistant transfected cells (6). The RNA was fractionated on 1% agarose gels, transferred to nylon membranes, and hybridized with a ^{32}P-labeled rat urate oxidase cDNA probe (1). For immunoblotting, whole-cell lysates adjusted to an equal amount of protein (65 μg) were solubilized in sample loading buffer, separated by SDS/PAGE, and transferred to nitrocellulose membrane. Immunodetection of urate oxidase was performed with specific polyclonal antibodies raised against recombinantly expressed rat urate oxidase (2).

Measurement of Uric Acid and Allantoin

Cells were treated with 0.5 mM uric acid for 4 days. Aliquots of culture medium were collected daily and treated with 5% trichloroacetic acid to remove serum proteins. Uric acid levels were then determined spectrophotometrically by monitoring the absorption at 292 nm (13). Allantoin was estimated by the Rimini-Schryver reaction (32).

Immunomorphological Procedures

Immunofluorescence localization of urate oxidase was performed using polyclonal antibodies raised against rat urate oxidase (2). Fluorescein-conjugated anti-rabbit antibody (Sigma) was used to visualize antigen–antibody complexes. Electron microscopy and immunogold localization procedures were the same as described previously (2,24).

Purification of Recombinantly Expressed Urate Oxidase

Purification of urate oxidase was accomplished by treating the cells with sodium deoxycholic acid as described elsewhere (2). Stably transfected CV-1 cells expressing rat urate oxidase (CV-UOX2 cells) were collected from 50 culture dishes and lysed in 10 mM Tris-HCl (pH 7.5) containing 2% sodium deoxycholate (2); they were stirred for 1 h at 4°C. This suspension was then centrifuged at 12,000 × *g* for 30 min and washed twice with the same buffer to eliminate possible membrane protein contamination. The pellet, which contained essentially all urate oxidase cores, was solubilized by stirring with 0.1 M Na_2CO_3 (pH 11) for 1 h (18). The purity of the protein was checked by SDS/PAGE. The enzyme activity of purified protein was determined by measuring the rate of consumption of uric acid (13). The isolated urate oxidase cores were also processed for electron microscopy.

RESULTS

Characterization of Stably Transfected CV-1 Cells Expressing Rat Urate Oxidase

CV-1 cells were transfected with an expression vector containing the full-length cDNA for rat urate oxidase under the control of cytomegalovirus promoter. As the vector also contained hygromycin-resistant gene, transformants were selected by treating the transfected cells with hygromycin. Five stably transfected clones were isolated and subcultured. To ascertain the presence of the exogenous rat urate oxidase cDNA, both Southern blot and PCR analysis were performed to demonstrate the integration of the transfected gene in these stable transfectants. As shown in Fig. 1, CV-1 cells had no endogenous urate oxidase expression (lane 1 in Fig. 1B and C), whereas four of the five hygromycin-resistant clones exhibited high levels of urate oxidase mRNA (Fig. 1B) and protein (Fig. 1C). Clone number two, designated CV-UOX2, strongly expressed rat urate oxidase mRNA and urate oxidase protein, as ascertained by Northern and immunoblotting, respectively (lane 3, in Fig. 1B and C).

Immunomorphological Features of CV-1 Cells Expressing Rat Urate Oxidase

In species that express urate oxidase, this protein is localized subcellularly within the peroxisomes of liver parenchymal cells and occasionally in peroxisomes present in the proximal tubular epithelium of kidney (25,26). Targeting of proteins to peroxisomes is directed by the consensus sequence of three amino acids Ser-Lys-Leu (SKL) at the carboxy-terminus of the protein (11). This tripeptide, or a conserved variant such as Ser-Arg-Leu present in rat urate oxidase (21), is both necessary for the sorting of proteins destined for intraperoxisomal localization and sufficient for directing normally cytosolic passenger proteins to peroxisomes (11). Although the recombinantly expressed rat urate oxidase formed discrete 1-3-μm diameter granular aggregates in insect Sf9 cells, the expressed protein could not be demonstrated in peroxisomes due to the absence or paucity of peroxisomes in Sf9 insect cells (2). To investigate whether rat urate oxidase would be targeted to the peroxisomes of primate epithelial cells, we stably transfected CV-1 cells. When these CV-UOX2 cells stably expressing recombinant rat urate oxidase were examined by immunofluorescence, numerous intensely fluorescent discrete granular bodies were found in the cytoplasm (Fig. 2A). No such staining was visualized in untransfected CV-1 cells (Fig. 2B) or in CV-UOX2 cells immunostained without the use of primary antibodies against urate oxidase. Transmission electron microscopic examination of the CV-UOX2 cells revealed electron dense structures/inclusions within several single membrane-limited cytoplasmic organelles (Fig. 3A); no such structures were detected in normal, untransfected CV-1 cells (Fig. 3B). These dense inclusions, seen exclusively in CV-UOX2 cells expressing recombinant urate oxidase, are composed of bundles of hollow tubules. Cytoplasmic organelles containing these tubular crystalloid cores (Fig. 4A and B) were subsequently identified as peroxisomes by using protein A-gold immunocytochemistry to visualize the presence of the peroxisomal marker enzyme catalase. These cores were found to display parallel-packed structures in the longitudinal section (Fig. 4C) and a honeycomb structure in the cross section (Fig. 4D). The orderly oriented circular structures consisted of unit bundles, the outer and inner diameters of which are approximately 150 and 50 Å, respectively. Ten circular bundles, each of which approximated its wall with adjacent bundles, are arranged forming a unit that appeared the same as that found in the urate oxidase-containing peroxisomes of rat liver (23). The space in the center of the unit outlined by 10 circles is slightly larger than the diameter of the component circles and contains a dense inclusion (Fig. 4D). The circles outlining the 10-member unit also serve

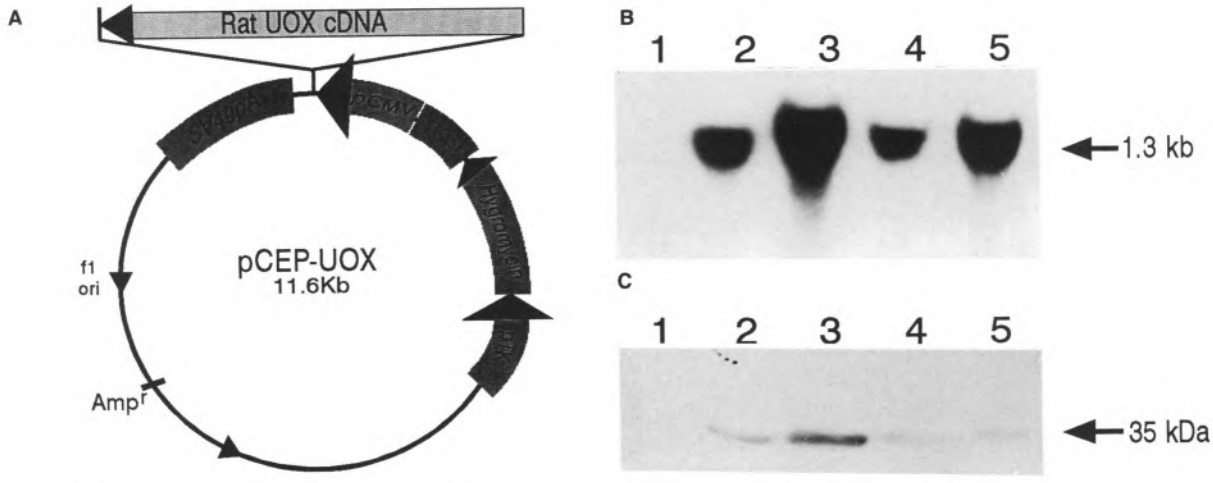

FIG. 1. Characterization of stably transfected CV-1 cells expressing rat urate oxidase. (A) The construction of an eukaryotic expression vector pCEP-UOX. The full-length rat peroxisomal urate oxidase cDNA was cloned into a Pvu II site of vector pCEP4, under the transcriptional control of the CMV promotor. (B) Northern blot analysis for expression of urate oxidase mRNA: lane 1: untransfected CV-1 cells, lanes 2–5: clone 1, 2, 3, and 5, respectively. Each lane was loaded with 15 μg total RNA and probed with ^{32}P-labeled rat urate oxidase cDNA. Arrow indicates the position of urate oxidase mRNA. (C) Western blot analysis for expression of urate oxidase protein. Cellular proteins (65 μg) from each cell lane as indicated in panel (B) were separated by SDS-PAGE, transferred to nitrocellulose filters, and stained with a specific antibody raised against rat urate oxidase. Arrow indicates the 35 kDa urate oxidase band.

as circles for adjacent 10-member units in the recombinantly expressed urate oxidase. This configuration is similar to that encountered in urate oxidase cores of rat liver (12,23).

Immunocytochemical staining for urate oxidase was strongly positive in CV-UOX2 cells stably expressing the recombinant rat urate oxidase (Fig. 5A). Labeling by gold particles is limited to the peroxisomes (Fig. 5A). The labeling appeared very intense with numerous gold particles essentially decorating the entire peroxisome in these CV-UCOX2 cells (Fig. 5A and B). The protein A-gold immunocytochemical staining using specific antibodies raised against urate oxidase failed to show specific labeling in untransfected CV-1 cells (Fig. 5C). Untransfected CV-1 cells and CV-UCOX2 cells expressing recombinant urate oxidase were weakly positive for catalase. It is of interest to note that catalase staining showed a different pattern in urate oxidase-expressing and -nonex-

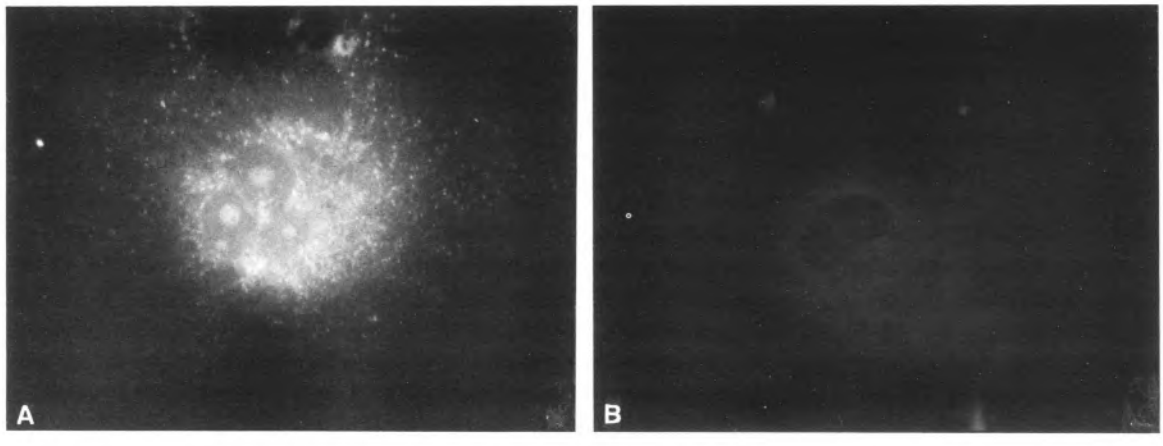

FIG. 2. Immunofluorescence staining of CV-UOX2 cells with anti-rat urate oxidase antibody. Indirect immunofluorescence appearance of CV-UOX2 cells (A) and untransfected CV-1 cells (B). Note the presence of numerous punctate granules in the cytoplasm of CV-UOX2 cells.

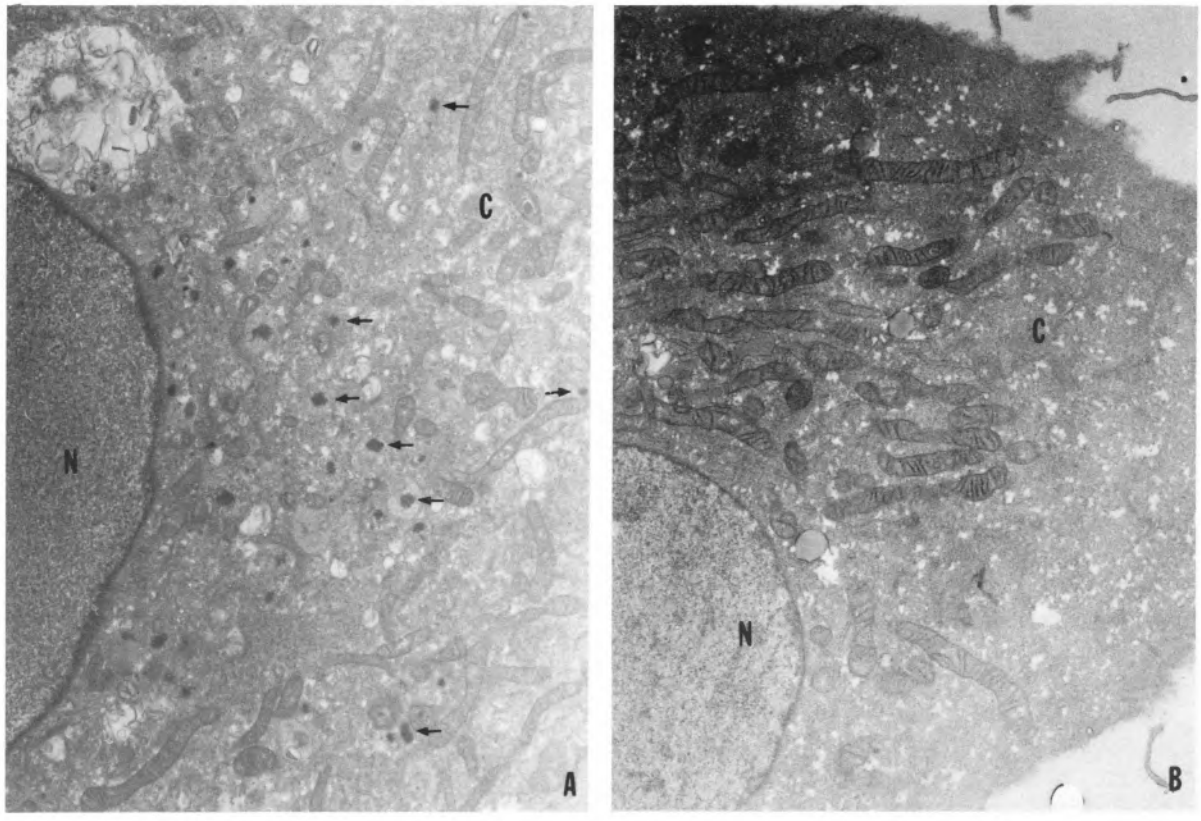

FIG. 3. Electron microscopic features of CV-UOX2 and CV-1 cells. Transmission electron micrographs of CV-UOX2 (A) and CV-1 cell (B). Numerous electron dense granules are observed in the cytoplasm of CV-UOX2 cell (arrows). N: nucleus; C: cytoplasm. Magnifications: ×8400.

pressing CV-1 cells (Fig. 5D and E). Catalase is distributed evenly in the peroxisome matrix of untransfected CV-1 cells (Fig. 5E), whereas catalase is present only at the periphery of the urate oxidase-containing crystalloid inclusion, as this inclusion essentially occupies the entire peroxisomal matrix of CV-UOX2 cells (Fig. 5A–D) and apparently displaces catalase to the periphery (Fig. 5D).

Conversion of Uric Acid to Allantoin In Vitro by CV-UOX2 Cells

Urate oxidase catalyzes the oxidation of uric acid to allantoin with molecular oxygen as the only known acceptor and in the process generates hydrogen peroxide (19,20). To investigate whether the CV-UOX2 cells are capable of this reaction in vitro, uric acid was added to the culture medium at a concentration of 0.25 mM. The consumption of this substrate was measured daily by monitoring absorption of the medium at 295 nm. As shown in Fig. 6A, significant reduction of uric acid was seen with CV-UOX2 cells. At day 4, the absorbance was decreased to the baseline level. With CV-1 cells, the substrate concentration changed only minimally (Fig. 6B). Thus, the CV-UOX2 cells expressing rat urate oxidase are able to convert uric acid to allantoin in vitro, but CV-1 cells failed to do so.

Purification of Rat Urate Oxidase From CV-UOX2 Cells

To purify the recombinantly expressed rat urate oxidase, CV-UOX2 cells were lysed using 2% sodium deoxycholate in 10 mM Tris-HCl (pH 7.4). After centrifugation at 12,000 × g, >95% of urate oxidase was pelleted as insoluble aggregates. After washing extensively with the same solution and repelleting, the precipitate was found to contain only urate oxidase, as indicated by SDS-PAGE analysis (Fig. 7A). The pellet, when processed for transmission electron microscopy, exhibited the same crystalloid structures as found in the CV-UOX2 cells (Fig. 7B). The pellet contained essentially all of urate oxidase and dis-

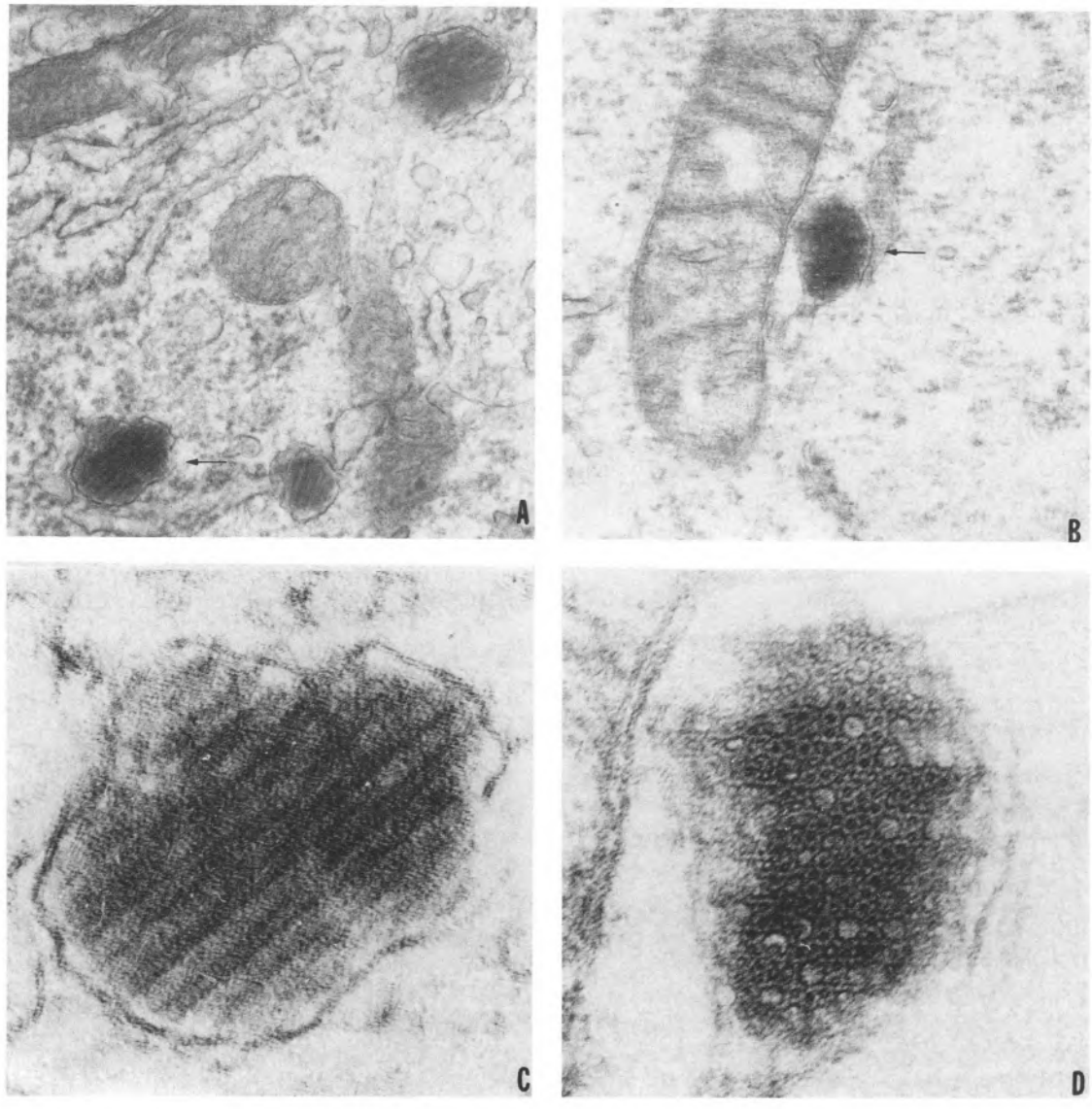

FIG. 4. Ultrastructural features of recombinant rat urate oxidase expressed in CV-UOX2 cells. Longitudinal (A, C) and cross (B, D) sections of the crystalloid core of rat urate oxidase in peroxisomes of CV-UOX2 cells. Magnifications: (A, B) ×45,000; (C, D) ×185,000.

solved in 0.1 M Na_2CO_3 at pH 11. The specific activity of the purified protein solubilized at pH 11 was 8.1 units per mg of protein, which was increased approximately 100 times through purification compared to that found in the whole-cell lysates (data not presented).

DISCUSSION

This study was undertaken to determine whether recombinantly expressed rat urate oxidase in a primate cell system can be targeted to the peroxisome and if it can form distinct crystalloid structures within such organelles. In rats and most other mammals, urate oxidase is localized exclusively within the peroxisomes of hepatic parenchymal cells and is present as a distinct peroxisomal core or nucleoid described as crystalline, crystalloid, or multilamellated core (8,16,24). There had been considerable controversy as to whether urate oxidase forms the crystalline core by itself (12), or whether such cores are formed by other protein(s) in which urate oxidase is simply compartmentalized (8,18). Hruban and Swift (12) demonstrated that rat liver peroxisome crystalloid cores are simi-

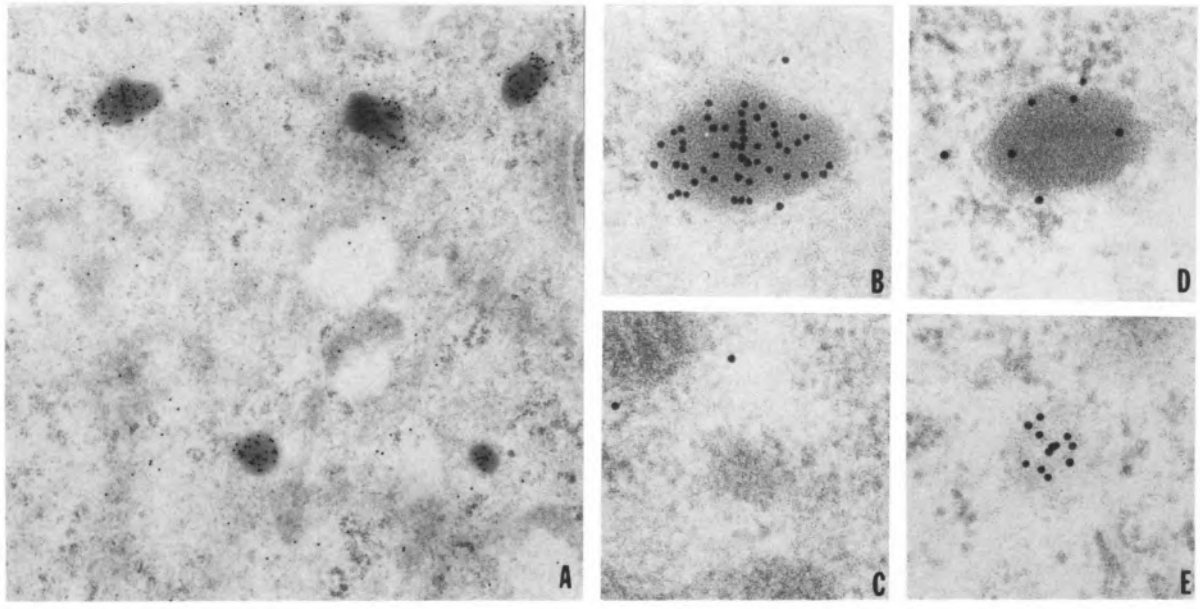

FIG. 5. Immunocytochemical localization of urate oxidase and catalase in CV-1 and CV-UOX2 cells by protein A-gold method. CV-UOX2 cells (A, B, D) and CV-1 cells (C, E) were immunostained using anti-rat urate oxidase antibodies (A, B, C) or anti-rat catalase antibodies (D, E), respectively. Note that urate oxidase is present in CV-UOX2 cells. Catalase is seen in both CV-UOX and CV-1 cells.

lar to the crystals of commercial preparations of porcine urate oxidase and surmised that cores are made up entirely of urate oxidase. This assumption was questioned based on subcellular fractionation data, and it was proposed that urate oxidase contributes no more than 25% of the protein of the cores in rat liver peroxisomes and that these cores contain other components (18). In an earlier study we attempted to resolve this controversy by expressing recombinant rat urate oxidase using baculovirus insect cell expression system (2). Although the peroxisomal targeting was not discerned, the results established that rat urate oxidase is capable of forming insoluble large crystalloid core-like structures. The baculovirus insect cell system was optimal for overexpression of urate oxidase, but it was not suitable for ascertaining peroxisomal targeting of urate oxidase. The insect cell lysis occurring 3 or 4 days after transfection with the recombinant baculovirus makes it difficult to establish stably transfected cell lines for additional investigations. Thus, the present study, using African green monkey kidney cells (CV-1 cells), was necessary to demonstrate peroxisomal targeting and crystalloid-core formation of recombinantly expressed urate oxidase in these cells that do not have such crystalloid structures.

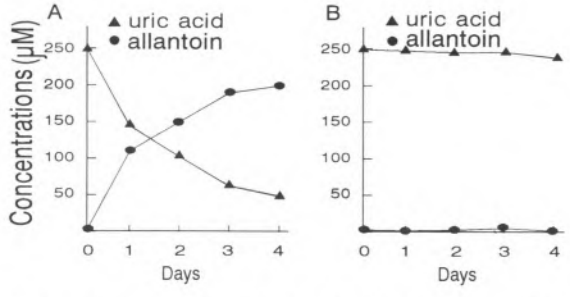

FIG. 6. In vitro conversion of uric acid to allantoin by stably transfected CV-UOX2 cells. A 10-mM uric acid stock solution was added to the culture medium of CV-UOX2 cells (A) and the control CV-1 cells (B). The degradation of uric acid and the production of allantoin were monitored daily by methods described in the Materials and Methods section.

Urate oxidase cores in rat liver peroxisomes contain dense parallel lines in the longitudinal section, and a honeycomb appearance in cross section (12,23). In the cross section the crystalloid structure was seen to contain less dense areas of two different sizes, 45 and 115 Á, arranged in such a way that each larger area was surrounded by a ring of 10 smaller areas. In the present study, a similar pattern was reproduced in peroxisomes of CMV-UOX2 stable transfectants expressing urate oxidase. These electron dense bodies in stably transfected cells are composed of hollow tubular structures with an internal diameter of 50 Å. Protein A-gold immunocytochemical studies con-

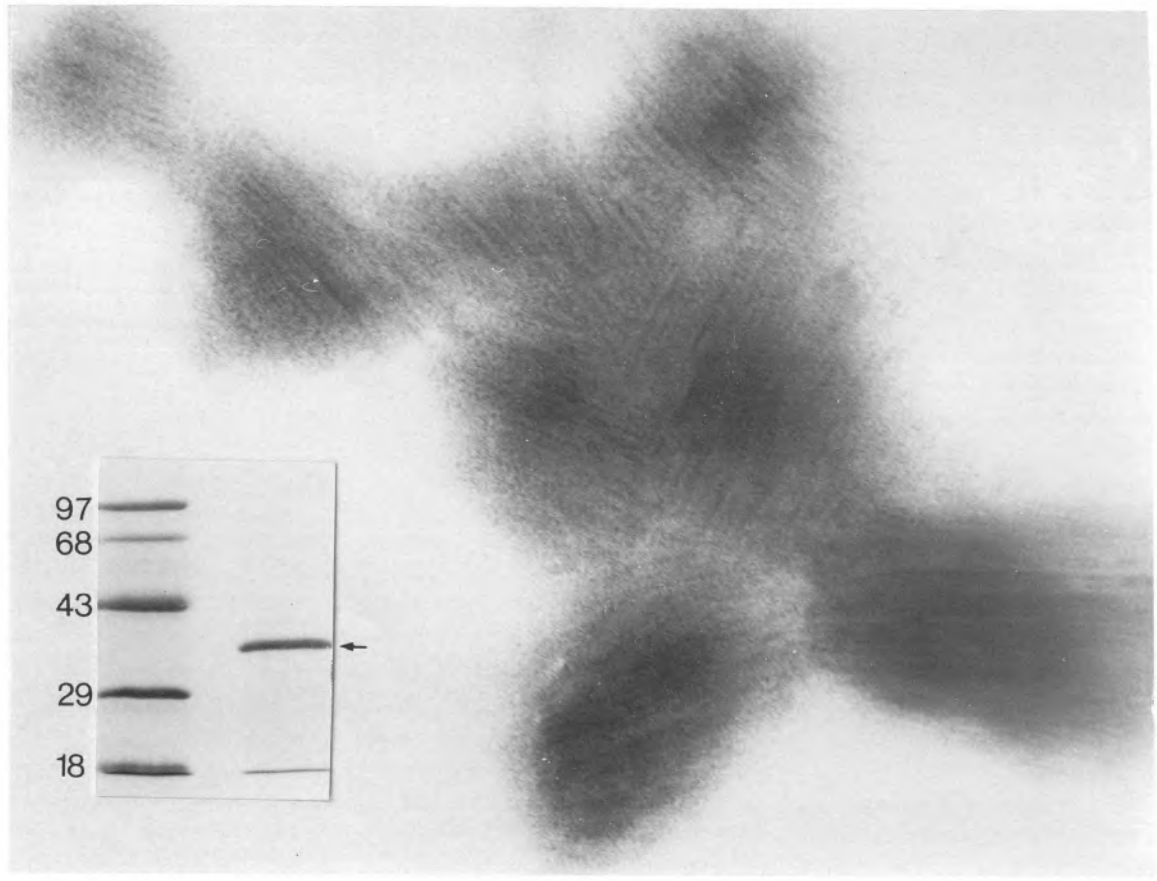

FIG. 7. Purification of recombinant rat urate oxidase from CV-UOX2 cells. CV-UOX2 cells were treated with 10 mM Tris-HCl (pH 7.5) containing 2% sodium deoxycholic acid. Soluble proteins were removed by spinning at 12,000 × *g* for 30 min and the pellet was washed twice with the same solution. The insoluble proteins were processed for transmission electron microscopy. The purified recombinant rat urate oxidase cores display the characteristic longitudinal tubules. SDS-PAGE of the isolated cores reveals the 35 kDa urate oxidase (inset).

firmed the tubular structures to be urate oxidase, where numerous gold particles were distributed over the tubular structures. Electron microscopy also confirmed the presence of these urate oxidase cores in cytoplasmic organelles measuring up to 3 μm in size and limited by a single membrane. These organelles in CMV-UOX2 cells containing the recombinant rat urate oxidase cores were identified as peroxisomes by demonstrating immunocytochemically the presence of the peroxisomal marker enzyme catalase.

The targeting of recombinantly expressed urate oxidase to peroxisomes in CV-1 cells with its distinct morphological appearance provides a model system for the elucidation of the role of peroxisomal targeting signal (PTS), and the translocation of newly synthesized protein into the peroxisomal matrix, in the oligomerization and crystalloid formation of this protein. A tripeptide peroxisomal targeting signal, Ser-Lys-Leu, or a conserved variant at the C- terminal region of the protein, designated PTS1, is necessary for sorting cytosolic proteins into peroxisomes (22). This peroxisomal targeting signal is shown to be evolutionarily conserved in diverse organisms (15). The C-terminal location of the PTS1 sequence has been confirmed directly for over 12 peroxisomal proteins, including urate oxidase (22). Other peroxisomal proteins, such as 3-ketoacyl CoA thiolases, which lack a C-terminal PTS1 sequence, possess an 11-amino acid sequence at the amino-terminus that also directs proteins to the peroxisome matrix (22). This amino-terminal PTS is referred to as PTS2. The existence of these targeting signals would indicate the presence of a receptor(s) capable of recognizing proteins with the PTS1 and/or PTS2. In our previous study, when we expressed rat urate oxidase in a baculovirus-insect cell system, the recombinant protein formed large insoluble crystalloid structures similar to rat urate oxi-

dase both in the cytoplasm and the nucleus of the insect cells (2). It is possible that there are few or no peroxisomes and/or receptors to recognize the PTS within the insect cells. In addition, the baculovirus expression system can overwhelm the cell due to the abundance of the recombinant urate oxidase leading to the formation of crystalloid cores randomly in the cell. In the present study, we expressed urate oxidase in a mammalian cell system with the intent to target the protein to peroxisomes. This study unequivocally demonstrates that the recombinant urate oxidase produced by stably transfecting a rat urate oxidase cDNA in CV-1 cells can be targeted efficiently to peroxisomes and that it forms crystalloid core structures resembling those present in rat liver peroxisomes. Urate oxidase, like some other peroxisomal proteins, has FAD prosthetic groups and it is believed that monomeric subunits are translocated into the peroxisome matrix where they are assembled with other subunits and oligomerized (17). Nevertheless, recently it has been demonstrated that prefolded proteins can translocate into the peroxisomal matrix and that protein unfolding is not a prerequisite for peroxisomal import (27). The model system described here will enable further studies to ascertain whether mutant urate oxidase monomeric subunits lacking the C-terminal PTS1 would assemble to form a crystalloid core outside the peroxisome in the cytosol.

The present study also revealed that the urate oxidase expressed in CV-1 cells is enzymatically active as ascertained by monitoring the culture medium for the conversion of uric acid into allantoin. Because this approach is simple, sensitive, and inexpensive, it is proposed that the urate oxidase gene can be used as a reporter gene for monitoring promoter activity. However, studies are required to compare the relative advantages of using the urate oxidase gene over the frequently used firefly luciferase gene (9) and bacterial chloramphenicol acetyltransferase gene (10).

Of great interest, however, is that the present study also provides cell lines stably expressing rat urate oxidase and such cell lines can serve as valuable tools to explore the role of overexpression of urate oxidase in hepatocarcinogenesis. In this context, it is important to note that a stably transfected CV-1 cell line overexpressing rat peroxisomal fatty acyl-CoA oxidase, when exposed to a fatty acid substrate for 2–6 weeks, formed transformed foci, grew efficiently in soft agar, and developed adenocarcinomas when transplanted into nude mice (7). These observations indicate that sustained overexpression of H_2O_2-generating fatty acyl-CoA oxidase causes cell transformation. The CV-1 cells stably expressing rat urate oxidase established in the present study will be valuable in confirming and extending the concept that sustained overexpression of H_2O_2-generating oxidases can play a role in carcinogenesis. It would be important to culture the CV-1 cells stably overexpressing urate oxidase in the presence of uric acid, a substrate for this enzyme, the conversion of which results in the generation of H_2O_2. Genetic damage could result from the H_2O_2 escaping out of the peroxisomal network. There is considerable evidence that cellular generation of reactive oxygen species, in particular hydroxyl radicals and singlet oxygen and the resulting oxidative damage to the nuclear DNA, participate in the process of mutagenicity, carcinogenicity, aging, and development of degenerative diseases. The CV-1 cells overexpressing fatty acyl-CoA oxidase (7), urate oxidase as reported in the present study, as well as other such transgenic cells and transgenic animals that overexpress H_2O_2-generating oxidases could serve as useful tools for elucidating the molecular events involved in transformation resulting from oxidative stress.

ACKNOWLEDGEMENTS

This work was supported by the Veterans Administration Merit Review grant to A.V.Y. and National Institutes of Health Grant R37 GM23750 to J.K.R.

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