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Hamster Hepatic Nuclear Mixed-Function Amine Oxidase: Location and Specific Activity

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SUMMARY

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Nuclei isolated from hamster liver contain activities characteristic of the mixed-function amine oxidase. The enzyme present in nuclei, like the microsomal monooxygenase, catalyzes the oxygenation of both nitrogen and sulfur compound. Activity per milligram protein of isolated nuclei is 12-14% that of microsomes. The hamster liver monooxygenase cross-reacts with antibodies raised in rabbits to the homogeneous pig liver microsomal mixed-function amine oxidase, and isolated nuclei stained with fluorescent labeled antibody indicate that the monooxygenase is largely concentrated in the nuclear envelope.

Metabolic transformations of drugs and xenobiotics at the nuclear level have recently received considerable attention. A number of investigations have shown that the endoplasmic reticulum is not the exclusive realm of aryl hydrocarbon hydroxylase (AHH), but that this enzyme is also found in the nucleus (1-4). Rogan *et al.* (5, 6) have reported that the rat hepatic nucleus contains cytochrome *P*-450 and that this enzyme can mediate a wide variety of biotransformations.

Prior to 1962, when Baker and Chaykin (7) suggested that an enzyme other than cytochrome *P*-450 was responsible for *N*-oxidation of trimethylamine, it was generally considered that cytochrome *P*-450 was the enzyme responsible for *N*-oxidation of amines. Mixed-function amine oxidase (MFAO),[1] another microsomal enzyme, has since been shown to participate in the oxidative metabolism of certain sulfur-containing compounds and secondary and tertiary amines (8-11). This enzyme is a flavoprotein (15) and its physiological function appears to be the generation of oxidizing equivalents necessary for disulfide bond formation during protein synthesis (16).

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[1] Abbreviations used: MFAO, mixed-function amine oxidase; TEMPO, 2,2,6,6-tetramethylpiperidinoxyl; DMA, *N*,*N*-dimethylaniline; MI, methimazole.

In this report, we describe experiments which demonstrate that mixed-function amine oxidase is present in hamster hepatic nuclei and that this enzyme is associated with the nuclear envelope. We also have shown that its activity and selectivity toward two substrates mimic those observed in microsomal preparations. Finally, we have determined that cytochrome *P*-450 is both present and active in hamster hepatic nuclei.

Male Syrian golden hamsters (70-110 g) obtained from GIBCO Animal Resources Laboratories were used in all experiments. Liver nuclei were prepared by a modification of the method of Bresnick *et al.* (4) as follows: The animals were sacrificed and then their livers were perfused through the hepatic portal vein with 50-70 ml of ice-cold buffer consisting of 50 mM Tris, 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$ (TSKM) at pH 8.3. Their livers were rapidly excised, the gall bladders were removed, and, after chilling in TSKM buffer, the livers were forced through a wire-mesh tea strainer with a Teflon pestle. They were then ground with one slow pass by hand of a Teflon pestle in a loose-fitting glass homogenizer. The homogenates were diluted to about 30 ml with TSKM buffer and centrifuged at 120*g* for 8 min to pellet the whole cells, erythrocytes, and clumps of cells. The supernatants were centrifuged at 2500*g* to pellet the nuclei. The nuclear pellets were resuspended in 30 ml each of 2.4 M sucrose containing 3 mM $CaCl_2$ and centrifuged at 35,000*g* for 50 min. The pellicles, consisting of the last

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remaining red blood cells, whole cells, and clumps, were discarded together with the clear yellow supernatants. The buff-colored nuclear pellets were washed once by resuspension in 25 ml of 1 M sucrose containing 1 mM $CaCl_2$ and centrifuged at 2500*g* for 10 min. The nuclei required for enzyme activity measurements were resuspended in TSKM buffer that had been passed through Bio-Rad Chelex-100 resin to remove divalent cations.

The final nuclear pellets were fixed for 1 h at room temperature in 4% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, postfixed in OsO_4, *en bloc* stained with uranyl acetate, then dehydrated and embedded in Epon 812. Thin sections were stained with lead citrate and uranyl acetate, carbon coated, and photographed with an Hitachi HUIIE transmission electron microscope.

Localization of the mixed-function amine oxidase in nuclei was determined by the indirect fluorescent antibody labeling method described by Coons (17). The nuclear pellets were transferred without dilution onto the microtome specimen holder, frozen in liquid nitrogen, and sectioned at 4 μm, and the sections were transferred to glass slides. After mounting, all specimens were first treated with 1% BSA for 10 min to decrease nonspecific binding of fluorescent labeled antisera. Excess BSA was removed by washing with 150 ml of 0.15 M NaCl in 0.01 M potassium phosphate, pH 7.0. The experimental plates were then treated with the primary antiserum (rabbit IgG against hog liver microsomal mixed-function amine oxidase) in a humidity chamber for 30 min. The control plates were incubated with rabbit serum freed of primary antiserum by titration with the purified monooxygenase. After washing with 150 ml of phosphate-buffered saline, the specimens were incubated with the secondary anti-

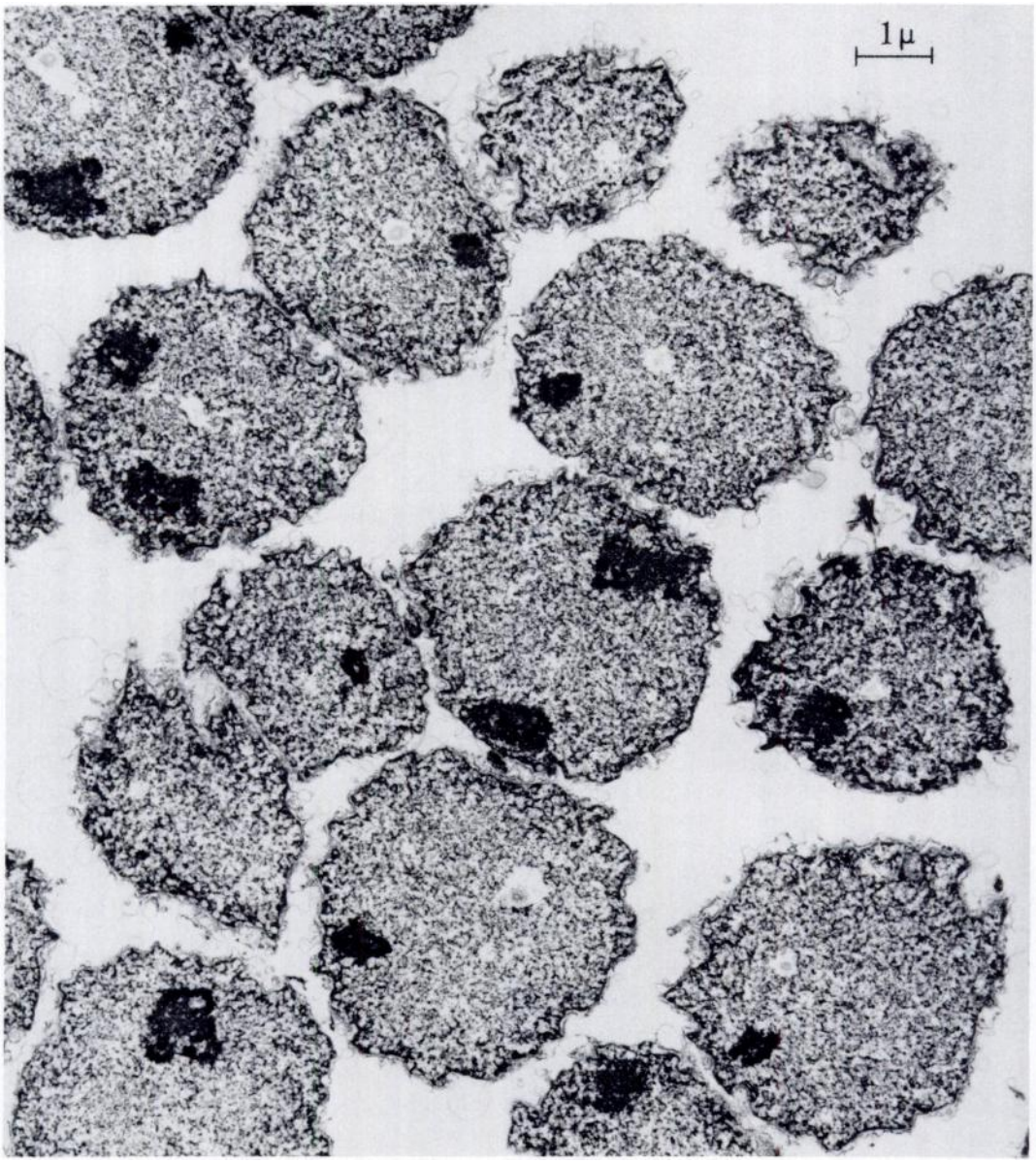

FIG. 1. *Isolated hamster liver nuclei*

Intact nuclei with one or more nucleoli and attached nuclear envelope are presented. Whole cells and contaminating cytoplasmic organelles are not seen. ×10,200.

serum (rhodamine-labeled goat anti-rabbit IgG) for 30 min. After washing to remove excess secondary antiserum, coverslips were mounted with glycerol. Selected fields were photographed with fluorescence and phase-contrast illumination. The final magnification including photographic enlargement was 1620×. Antibodies to the homogeneous pig liver mixed-function amine oxidase were prepared as described earlier (13). Anti-rabbit IgG raised in goats (Miles Biochemicals) was labeled with rhodamine as described by Brandtcagt (18).

Samples of hamster or hog liver were cooled in an ice bath containing 0.25 M sucrose. The tissue was trimmed to approximately 2 × 5 × 5 mm and mounted on a small piece of cork with Tissue Tek O.C.T. Compound (Scientific Products) and then frozen by immersion in liquid nitrogen. The frozen tissue block was transferred to an International Harris Model CT cryostat and sectioned at 3 to 4 μm. The sections were transferred to slides precoated with 0.5% gelatin in 0.001 M potassium sulfate and stained by the indirect fluorescent antibody labeling technique of Coons (17).

All sections were first treated at 20°C with 1% bovine serum albumin in normal saline to reduce nonspecific staining. After washing for 5 min with normal saline containing 0.01 M phosphate, pH 7.0, the sections were washed for 10 min to remove the excess serum and then covered with rhodamine-labeled goat anti-rabbit IgG (0.1 mg/ml). After 30 min of incubation, the sections were washed for 10 min to remove soluble serum and a coverslip was mounted with 90% glycerol in 0.01 M phos-

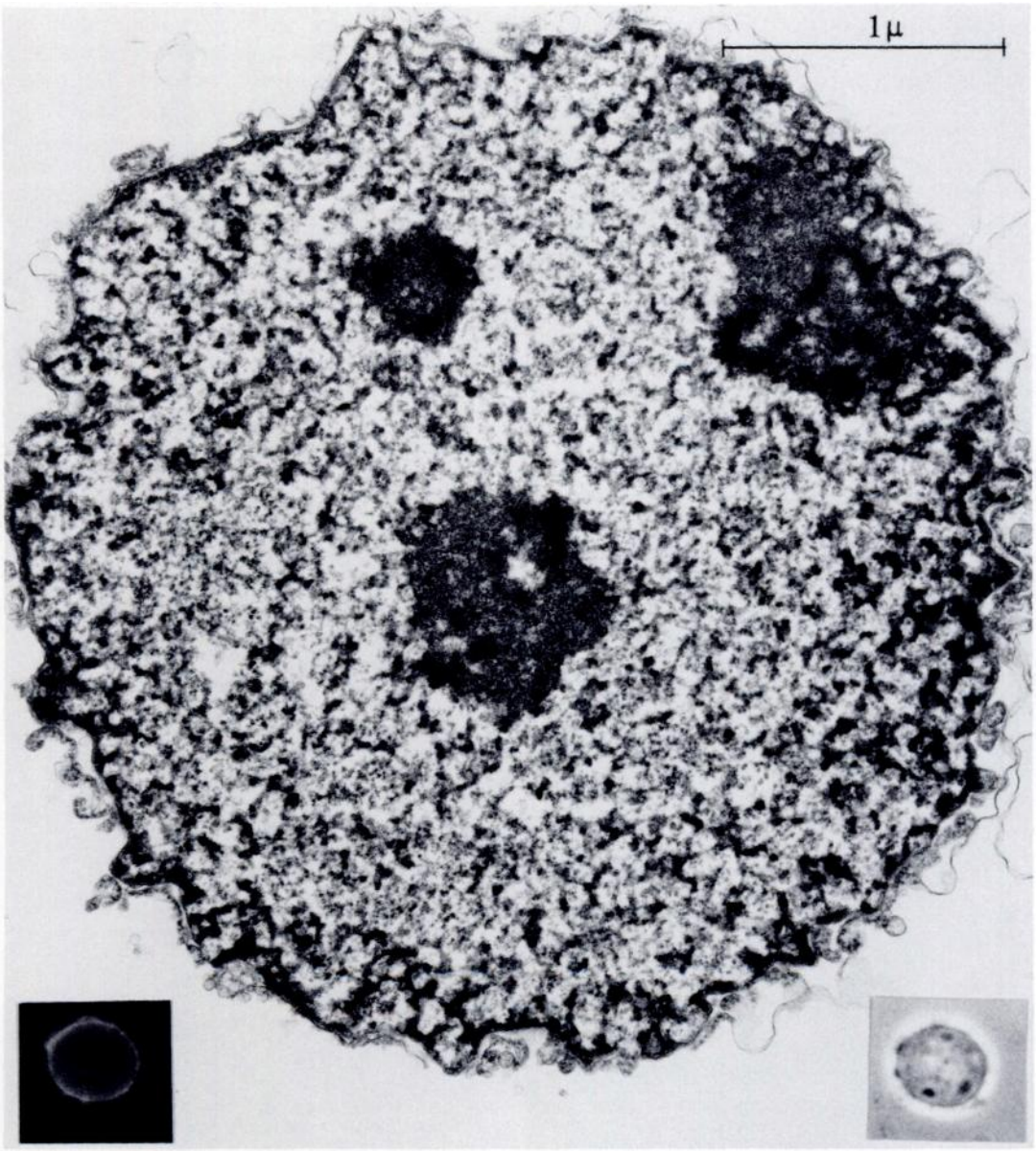

FIG. 2. *Isolated hamster liver nucleus*

This nucleus displays portions of three nucleoli and is enveloped by inner and outer nuclear membranes. The latter is studded with ribosomes and shows occasional discontinuities. ×38,000.

Left inset: Fluorescent photomicrograph of nucleus showing specific antibody fluorescence.

Right inset: Phase-contrast photomicrograph of nucleus shown at left.

phate, pH 7.0. The sections were examined with a Zeiss Universal microscope equipped with epifluorescent optics and planacromate objective lenses. Representative fields were photographed with fluorescent and phase-contrast illumination.

Mixed-function amine oxidase activity of isolated nuclei and microsomes was determined by measuring the rate of methimazole- or *N,N*-dimethylaniline-dependent NADPH oxidation with a Perkin Elmer 557 spectrophotometer in the double wavelength mode (λ_{obs} 340, λ_{ref} 420). The assay was run at 37°C, pH 8.3, with a saturating concentration of NADPH (0.1 mM) in 100 mM tricene buffer containing 2.4 mM *n*-ocytlamine, 1 mM diethylenetriamepentaacetic acid (Sigma Chemical Co.), and 1 mM EDTA. *n*-Octylamine has been reported to inhibit reactions catalyzed by cytochrome *P*-450 (19). Emulgen 913 detergents 0.5% (Kao-Atlas, Ltd., Tokyo) was added to prevent clumping of the nuclei. Hamster liver microsomes were isolated from perfused liver by a standard method (20).

Cytochrome *P*-450 activity was measured by following the rate of reduction of a stable nitroxyl radical, 2,2,6,6-tetramethylpiperidinoxyl (TEMPO), a substrate specific for the ferrous form of this hemoprotein (20). The kinetic studies were conducted using an epr spectrometer (Varian Associates Model E-9) to measure the decrease in the height of the central peak of the nitroxide triplet as a function of time. In a typical experiment, the reaction medium contained 10 μM nitroxide, 250 μM NADPH, 0.1 ml of nuclear suspension, and sufficient buffer (0.1 M phosphate with 1 mM diethylenetriaminepentaacetic acid adjusted to pH 7.4) to bring the final volume to 0.5 ml.

Cytochrome *P*-450 content was measured by the method of Omura and Sato (21).

All enzyme measurements were normalized to protein by the method of Bradford (22) using the dye reagent and standard bovine γ-globulin obtained from Bio-Rad Laboratories.

By electron microscopy, the pellets of hamster hepatic nuclei were observed to be free of whole cells, and separate or attached cytoplasmic organelles were not seen (Figs. 1 and 2). The nuclei contained up to three nucleoli in a single plane of section. They also contained occasional clear areas, which appeared to be pseudoinclusions from invagination of the nuclear membranes. The outer membrane, with its ribosomes, was uniformly present, although discontinuities were observed.

Indirect fluorescent staining of sectioned hamster nuclei with antibody specific for mixed function amine oxidase revealed that this antibody adhered to the nuclear membrane (Fig. 2). Controls, as described in the experimental section, showed no fluorescence. In order to define whether this localization might be an artifact of tissue fragmentation, frozen sections of hamster and hog liver were also stained with the fluoresecent antibody. Specific fluorescence demonstrated mixed-function amine oxidase with the cytoplasm of hepatocytes and bile duct epithelial cells. Enhanced fluorescence was observed at the nuclear membrane in both hamster and hog liver. Hepatocytes illustrating specific cytoplasmic fluorescence with nuclear membrane accentuation are shown in Fig. 3.

Mixed-function amine oxidase activity in nuclei is observed to be 14% of that in microsomes when assayed with *N,N*-dimethylaniline and 12.5% of that in microsomes when assayed with methimazole (Table 1). Within the limits of experimental error, both substrates give approximately the same ratio of mixed-function amine oxidase activity between nuclei and microsomes.

There is about four times as much cytochrome *P*-450 in microsomes as in nuclei as determined by its carbon monoxide difference spectrum. The activity of cyto-

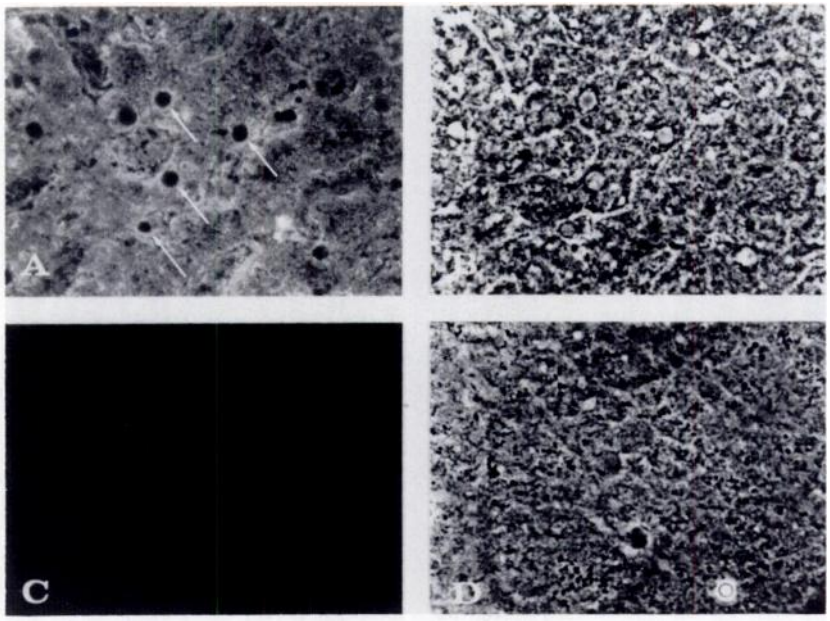

FIG. 3. *Porcine liver sections were treated with rabbit anti-MFAO IgG (A and B) or with rabbit IgG previously absorbed with MFAO (C and D), then with rhodamine-labeled goat anti-rabbit IgG*

A and C were photographed under fluorescent light, while B and D were photographed with phase-contrast illumination. Arrows indicate enhanced fluorescence at the nuclear envelope of hepatocytes. The original magnification was 1620.

TABLE 1

Distribution of mixed-function amine oxidase and cytochrome P-450 in microsomes and nuclei[a]

	MFAO activity		Cytochrome *P*-450	
	DMA	MI	TEMPO	Wavelength and content
	nmol/mg protein min	*nmol/mg protein min*	*nmol/mg protein min*	*nm and nmol/mg protein*
Microsomes				λ = 451
	6.6 ± 0.7	7.4 ± 0.7	3.4 ± 0.3	0.25 ± 0.02
Whole nuclei				λ = 453
	0.9 ± 0.2	0.9 ± 0.3	0.7 ± 0.2	0.07 ± 0.01
Whole nuclei/ microsomes	14%	12%	21%	28%

[a] The kinetic parameters were determined using liver organelles from eight groups of hamsters. These values are presented as means ± SE.

chrome *P*-450, as measured by TEMPO reduction (19), in microsomes was found to be about five times that in nuclei. We observed cytochrome *P*-450 absorption maxima of 453 nm for hamster hepatic nuclei and 451 nm for hamster hepatic microsomes.

Many carcinogenic compounds enter the body as procarcinogens, acquiring the ability to transform cells into neoplasias only after being themselves transformed into active metabolites. Cytochrome *P*-450 (23) and mixed-function amine oxidase (12) are different monooxygenases that apparently render certain compounds carcinogenically active. If so, their strategic location in the nuclear envelope would facilitate access of carcinogenic and mutagenic metabolites to DNA and minimize their chances of being detoxified in the cytoplasm before they can alter chromosomal integrity. The cytochrome *P*-450 system, which activates procarcinogens such as benzo-[*a*]pyrene, has recently been shown to be localized both on the nuclear surface (24) and within the nucleus (25).

Floyd and co-workers (26) have suggested that the procarcinogen, *N*-hydroxy-2-acetylaminofluorene, can be oxidized to its corresponding nitroxide by means of a one-electron transfer process. The biological oxidants superoxide and hydroxyl radical could mediate such oxidations. In previous publications, we showed that purified mixed-function amine oxidase generates superoxide (27) and that superoxide can oxidize hydroxylamines to nitroxides (28). With the observation that this enzyme is located in the nuclear envelope, the activation of procarcinogens by this flavoprotein takes on greater significance.

Identification of cytochrome *P*-450 and the flavin-containing monooxygenase known to be concentrated in the endoplasmic reticulum (29, 12) in nuclear preparations requires that the preparations be free of endoplasmic reticular comtamination. The electron micrographs demonstrate that the hamster liver nuclei used in these experiments were of suitable purity.

Two different techniques, fluorescent antibody staining and enzymatic activity, were employed to show that active flavin-containing monooxygenase is found in the nuclear envelope. Based on milligrams of total protein, nuclear preparations contained less mixed-function amine oxidase than microsomal preparations. However, approximately 97% of the total nuclear protein is associated with the chromatin, nucleoli, and nucleoplasm (30), whereas the fluorescent antibody staining indicates that mixed-function amine oxidase is localized primarily in the nuclear envelope. Thus, the specific activity of this enzyme in the nuclear envelope is probably greater than in the microsomes.

In conclusion, we have shown histochemically that the vast majority of nuclear mixed-function amine oxidase is restricted to the nuclear envelope, unlike cytochrome *P*-450, which has been found within nuclei (25) as well as on their outer surfaces (24). Furthermore, we have demonstrated that while the specific activity of mixed-function amine oxidase is probably higher in the nuclear envelope than in the endoplasmic reticulum, nuclear mixed-function amine oxidase mimics this enzyme in microsomal substrate specificity and antigenic properties. Whether this flavin-containing enzyme generates superoxide in the nuclear envelope, as observed for mixed-function amine oxidase in the endoplasmic reticulum (28), is currently under investigation.

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