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Hydroxymethylglutaryl-coenzyme A reductase-containing hepatocytes are distributed periportally in normal and mevinolin-treated rat livers

(cholesterol synthesis inhibitors/periportal hydroxymethylglutaryl-coenzyme A cell clusters/proliferation of smooth endoplasmic reticulum/immunofluorescence microscopy/electron microscopy)

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ABSTRACT **Mevinolin is a potent inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase; EC 1.1.1.34), an enzyme that catalyzes the rate-limiting step in cholesterol biosynthesis. We have been studying the hepatic distribution of reductase with immunofluorescence microscopy and liver ultrastructure with electron microscopy in normal and drug-treated rats. In control animals, only about 20% of the hepatocytes were reductase positive. These cells were localized in the periportal lobular zones. The numbers of positive hepatocytes in animals given mevinolin or cholestyramine (or both) were directly proportional to the activities of the HMG-CoA reductase determined biochemically. This induction of HMG-CoA reductase immunofluorescence was centered periportally. Rats given 0.075% mevinolin alone had a homogeneous distribution of reductase staining in their hepatocyte cytoplasm, whereas a combination of 0.25% mevinolin and 3% cholestyramine caused a 150-fold increase in enzyme activity and induced prominent juxtanuclear immunofluorescent globules of HMG-CoA reductase in all hepatocytes. With electron microscopy, these bodies were composed of tightly packed stacks of smooth endoplasmic reticulum cysternae and aggregates of branched smooth endoplasmic reticulum tubules. Our data suggest that a subpopulation of periportal rat hepatocytes may be uniquely specialized for cholesterol synthesis.**

Mevinolin, a fungal metabolite isolated from *Aspergillus terreus*, is a potent competitive inhibitor of the rate-limiting enzyme of cholesterol biosynthesis, microsomal 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase; EC 1.1.1.34) (1). A similar but somewhat less active inhibitor, compactin (ML-236B), has been purified from *Penicillium citrium* (2). These compounds are effective cholesterol-lowering agents in several species, including dogs (1, 3, 4) and humans (5–10) but not rats (11, 12). Furthermore, 50–70% reductions in the plasma cholesterol levels of dogs and humans have been obtained with combinations of bile acid sequestrants and mevinolin or compactin (4, 8–10).

The synthesis of HMG-CoA reductase in rat liver is exquisitely sensitive to a number of factors, including diurnal variation (13, 14). Treatment of rats with either an HMG-CoA reductase inhibitor or a bile acid sequestrant (or both) results in a marked elevation of enzyme level (14–17). This induction is the result of a dramatic increase in reductase mRNA levels and an enhancement of enzyme stability (15–17). Similarly, in UT-1 cells, a compactin-resistant line, reductase levels are greatly elevated but may be repressed abruptly by low density lipoprotein administration (18). Microscopic studies of these cells reveal a dramatic development of the smooth endoplasmic reticulum (SER) associated with high concentrations of HMG-CoA reductase (19–21).

The purpose of this study was to determine the hepatic distribution of HMG-CoA reductase and to correlate putative alterations in reductase patterns and ultrastructure with the extent of enzyme induction. Our results show that reductase-positive hepatocytes are localized unexpectedly in the periportal zones of untreated rat liver and that all hepatocytes are strikingly reductase positive following maximal enzyme induction.

MATERIALS AND METHODS

Materials. Mevinolin was purified from *A. terreus* at Merck as described (1). Cholestyramine and fluorescein isothiocyanate- or peroxidase-conjugated goat anti-rabbit IgG (IgG fraction) were obtained from Merck, Miles, or Bio-Rad, respectively.

Animals. Male Holtzman rats (200 g) were kept under normal (12 hr of light, 12 hr of dark) or reversed lighting. They were killed at 10 a.m., which is the diurnal low in normal lighting and the diurnal high in reversed lighting. Control rats were fed standard laboratory chow, whereas the experimental animals were fed ad lib with chow containing drugs on a wt/wt percentage basis.

HMG-CoA Reductase Antibody Production and Specificity. HMG-CoA reductase was purified to homogeneity from rat liver microsomes as described (22) and had a molecular mass of 5.5×10^4 daltons (due to proteolysis; Fig. 1, lane 2). Antisera were raised in rabbits and tested for specificity by using electrophoretic transfer blots. These antisera recognized only one protein within the entire microsomal fraction isolated in the presence of protease inhibitors (Fig. 1). This protein, at the 1×10^5-dalton position, represents uncleaved, native HMG-CoA reductase (23). Our HMG-CoA reductase antibodies are therefore monospecific.

Immunofluorescence (IF) Microscopy. Fresh unfixed liver blocks (0.5 cm^3) were mounted in embedding medium for frozen tissues (O.C.T. compound, Lab-Tek, Naperville, IL) and rapidly quenched in refreezing Freon 22 cooled by liquid nitrogen. Five-micron sections were cut on a cryostat at −23°C, stained with rabbit antiserum to HMG-CoA reductase diluted 1:80 with albumin/P_i buffer (0.1% globulin-free bovine serum albumin/0.1 M phosphate buffer, pH 7.8/0.1% NaN_3) followed by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG diluted 1:25 with albumin/P_i buffer. For

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Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; EM, electron microscopy; IF, immunofluorescence; SER, smooth endoplasmic reticulum; RER, rough endoplasmic reticulum.

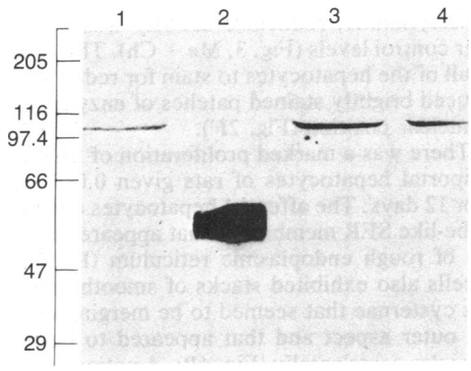

FIG. 1. Electrophoretic transfer blot of microsomal liver fractions showing monospecificity of our HMG-CoA reductase antibodies. Staining was performed with a 1:100 dilution of rabbit HMG-CoA reductase antiserum, followed by peroxidase-conjugated goat anti-rabbit IgG. Approximately 500 μg of microsomal protein was applied in lanes 1, 3, and 4. Microsomes were isolated in 0.25 M sucrose containing 40 μM leupeptin (lane 1), 5 mM EGTA (lane 3), or both (lane 4). In lane 2, 0.5 μg of purified homogeneous proteolytically modified reductase was applied. Molecular masses are shown in kilodaltons.

controls, normal rabbit serum or albumin/P_i buffer was substituted for the anti-HMG-CoA reductase serum. All control sections from normal or drug-treated rats were always completely unstained. Coverslips were mounted with 4% *n*-propylgallate in glycerol (24).

Electron Microscopy (EM). Blocks (0.5 mm^3) were fixed in 3.5% paraformaldehyde/2.0% glutaraldehyde/0.1 M sucrose/0.1 M phosphate buffer, pH 7.2. Then, fixation with 1% osmium tetroxide, embedment in Epon 812, and grid staining with uranyl acetate and Reynolds lead citrate (25) were performed.

RESULTS

IF Localization of HMG-CoA Reductase and Correlations with Induced Enzyme Levels. Untreated rats had clusters of HMG-CoA reductase-positive hepatocytes that were usually located in the periportal regions of the liver (Fig. 2*A*). Not all of the periportal hepatocytes were labeled, and they appeared to be randomly distributed surrounding the triad of artery, vein, and bile duct. The reductase staining filled the cytoplasm of these hepatocytes and exhibited a somewhat mottled pattern (Fig. 2*D*). About 18% of the hepatocytes were reductase positive at the low point in the diurnal rhythm of this enzyme (Fig. 3, U-N). This periportal pattern did not change at the diurnal high point, although 27% of the hepatocytes were stained and the HMG-CoA reductase activity was enhanced about 5-fold (Fig. 3, U-R). Liver sections from animals given 3% cholestyramine for 12 days exhibited the same intracellular staining pattern found in untreated rats and a similar periportal distribution of labeled hepatocytes. The main difference between these groups was that 36% of hepatocytes were HMG-CoA reductase positive; there was a similar 5-fold increase in enzyme activity following 12 days of cholestyramine treatment (Fig. 3, Ch-N). Rats treated with 0.025–0.075% mevinolin for 10–20 days dis-

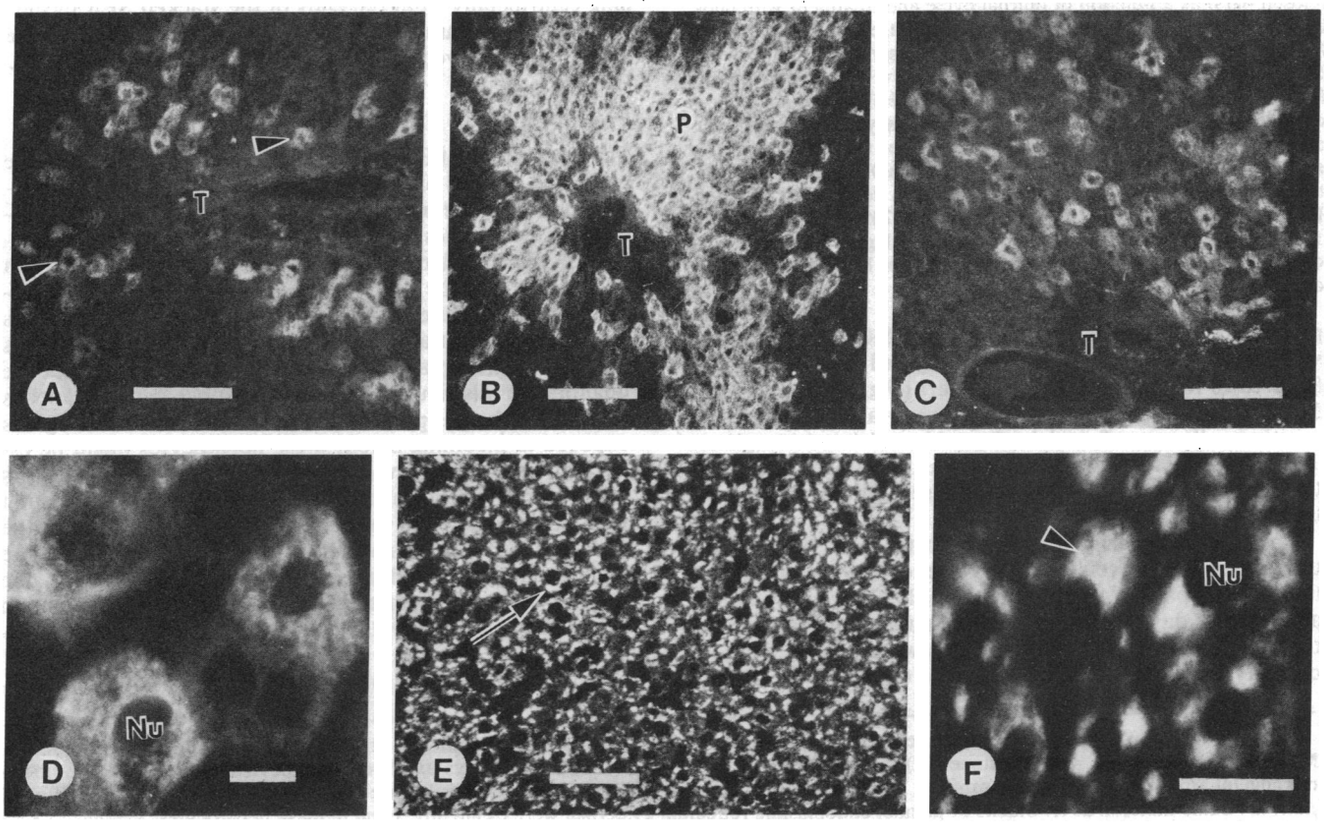

FIG. 2. IF microscopic distribution of HMG-CoA reductase in livers from normal or mevinolin-treated rats. (*A*) HMG-CoA reductase-containing hepatocytes (arrowheads) are scattered randomly about the portal triad (T) in untreated rats. (Bar = 100 μm.) (*B*) Mevinolin treatment (0.075%, 20 days) induces an intense staining for reductase in the periportal hepatocytes (P) surrounding the triad (T). (Bar = 100 μm.) (*C*) A normal pattern of HMG-CoA reductase localization is observed 8 days after cessation of the mevinolin regimen (0.075%, 12 days); T = portal triad. (Bar = 100 μm.) (*D*) A mottled pattern of reductase staining is distributed throughout the cytoplasm of untreated hepatocytes; their nuclei (Nu) are unstained. (Bar = 10 μm.) (*E*) All hepatocytes exhibit granules (arrow) of intense HMG-CoA reductase staining in rats given 3% cholestyramine for 9 days followed by 0.25% mevinolin and 3% cholestyramine for 3 days. (Bar = 50 μm.) (*F*) High magnification of hepatocytes from *E*. Brilliant globules of reductase (arrowhead) are found in a juxtanuclear location; Nu = nucleus. (Bar = 25 μm.)

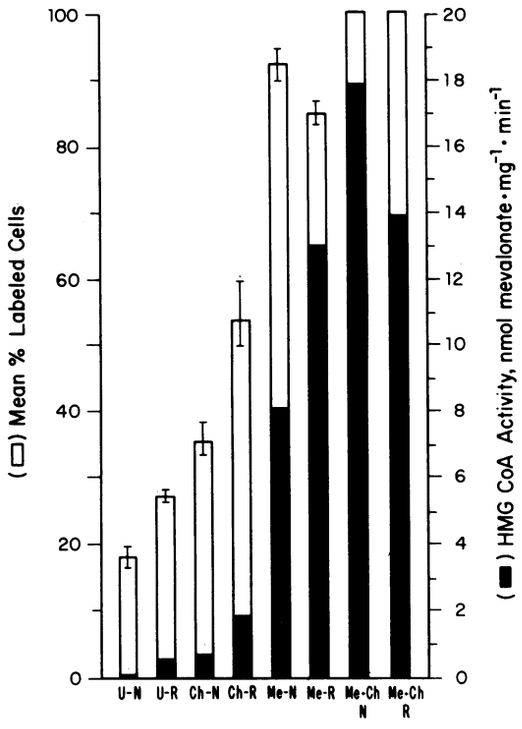

FIG. 3. Percentage of hepatocytes labeled for HMG-CoA reductase by IF microscopy (open bars) and levels of reductase activity (closed bars) as a function of diurnal cycle and treatment with cholestyramine or mevinolin (or both). U = untreated; N = normal lighting; R = reversed lighting; Ch = 3% cholestyramine for 12 days; Me = 0.075% mevinolin for 12 days; Me + Ch = 3% cholestyramine for 9 days followed by 0.25% mevinolin and 3% cholestyramine for 3 days. HMG-CoA reductase activities were assayed on pooled liver microsomes (three livers per group) as described (1). The assay buffer (0.1 M phosphate, pH 7.4) contained an NADPH-generating system. The mean % of labeled hepatocytes was determined by using IF micrographs encompassing a total area of 705 μm^2 representing 1890–2253 cells per group; brackets = SEM. All groups were significantly different except N versus R with Ch or Me treatment (Student's t test, $P < 0.05$).

played a highly significant induction of HMG-CoA reductase staining (80–92% labeled hepatocytes) and enzyme activity (a 30- to 70-fold increase) in their livers (Fig. 2*B*; Fig. 3, Me). Most of the periportal hepatocytes were labeled, and many positive cells were found in the midzonal lobular regions. The intracellular staining pattern was unchanged, although the hepatocytes appeared more intensely labeled than in untreated rats (Fig. 2 *A–C*).

In reversal experiments, the reductase activity had returned to normal levels by 2 days after drug withdrawal, and the staining patterns were indistinguishable from untreated animals at 8 days following mevinolin removal (Fig. 2*C*). In the most extreme case in which rats were primed with 3% cholestyramine followed by 3 days on 0.25% mevinolin and 3% cholestyramine, reductase activity was increased 150-fold over control levels (Fig. 3, Me + Ch). This drug regimen caused all of the hepatocytes to stain for reductase (Fig. 2*E*) and induced brightly stained patches of enzyme localized in a juxtanuclear position (Fig. 2*F*).

EM. There was a marked proliferation of SER in many of the periportal hepatocytes of rats given 0.075% mevinolin alone for 12 days. The affected hepatocytes contained masses of tube-like SER membranes that appeared to merge with profiles of rough endoplasmic reticulum (RER) (Fig. 4*A*). These cells also exhibited stacks of smooth-surfaced membranous cysternae that seemed to be merging with the RER at their outer aspect and that appeared to be budding off SER vesicles centripetally (Fig. 4*B*). A noticeable increase in the size and frequency of the SER stacks was found in the livers of rats given mevinolin for 20 days (not shown). Following 8 days of drug removal, this SER proliferation disappeared, and the hepatocytes displayed normal-looking parallel arrangements of RER profiles (Fig. 4*E*). Rats given the regimen of mevinolin and cholestyramine, which induced a dramatic 150-fold elevation in reductase levels (Fig. 3), exhibited the most impressive SER expansion. Large globular masses composed of expanded stacks of SER cysternae at their periphery and numerous anastomosing SER tubules located centrally were found in a juxtanuclear position in nearly every hepatocyte studied (Fig. 4*C*). These SER aggregates evidently correspond to the perinuclear globules of HMG-CoA reductase staining (Fig. 2 *E* and *F*). RER profiles also appeared to merge with outer facets of these SER stacks (Fig. 4*C*), and anastomosing SER tubules seemed to be budding centripetally from the ends of the stacked SER cysternae (Fig. 4*D*). Paracrystalline arrays of SER tubules were sometimes observed within these SER aggregates (Fig. 4*C*).

DISCUSSION

The localization of HMG-CoA reductase-positive cells in the periportal regions of normal livers, as well as in mevinolin- or cholestyramine-treated animals, suggests that the hepatocytes of these zones contain more reductase than centrilobular cells. This striking observation implies that periportal rat hepatocytes may be uniquely specialized for cholesterol synthesis. Cellular heterogeneity within the hepatic lobule has also been demonstrated for several other important enzymes (26, 27), for the uptake of a bile acid analog (28), and for the quantitative distribution of various subcellular organelles (29). Since the periportal areas are the first lobular regions exposed to incoming blood, these hepatocytes might be especially sensitive to low concentrations of putative effectors (e.g., bile acid, cholesterol, or mevalonate metabolite) that may occur locally. In the case of mevinolin treatment, the periportal hepatocytes would probably be exposed to the highest intralobular drug concentrations. Therefore, one might explain these periportal labeling patterns as a function of physiological gradients that probably exist in hepatolobular sinusoidal blood.

Our data also show that even though most of the hepato-

FIG. 4. (*on following page*) Electron micrographs of hepatocytes from rats given mevinolin with or without cholestyramine. Nu = nucleus; M = mitochondrion; Pr = peroxisome. (*A*) Rats treated with 0.075% mevinolin for 12 days exhibited hepatocytes with many SER tubules merging (at arrowheads) with RER profiles. The corresponding IF distribution of HMG-CoA reductase of this tissue is seen in Fig. 2*B*. (Bar = 1 μm.) (*B*) Same material as *A*. Stacks of parallel endoplasmic reticulum cysternae were frequently found in these hepatocytes. Profiles of RER appeared to be coalescing into these stacks (arrows), and SER vesicles (arrowheads) seemed to be budding from their ends. (Bar = 250 nm.) (*C*) A juxtanuclear SER aggregate often found in hepatocytes of rats given cholestyramine and mevinolin as in Fig. 2*E*. Wavy stacks of SER membranes are located peripherally and merge with RER cysternae at arrows. SER vesicles (arrowheads) fill the interior, and a paracrystalline SER array is also present (P). The IF localization of HMG-CoA reductase within similar hepatocytes is shown in Fig. 2*F*. (Bar = 500 nm.) (*D*) Portion of a hepatocyte SER aggregate from a rat treated with mevinolin and cholestyramine as described in Fig. 2*E*. Stacks of SER membranes found at the center of this aggregate apparently give rise to the numerous anastomosing SER tubules at their termini (arrowheads). (Bar = 250 nm.) (*E*) Ultrastructure of hepatocyte cytoplasm from a rat given 0.075% mevinolin for 12 days followed by 8 days of drug abstinence, as in Fig. 2*C*. These cells are filled with many parallel RER cysternae, lacked an observable SER, and were indistinguishable from untreated hepatocytes. (Bar = 500 nm.)

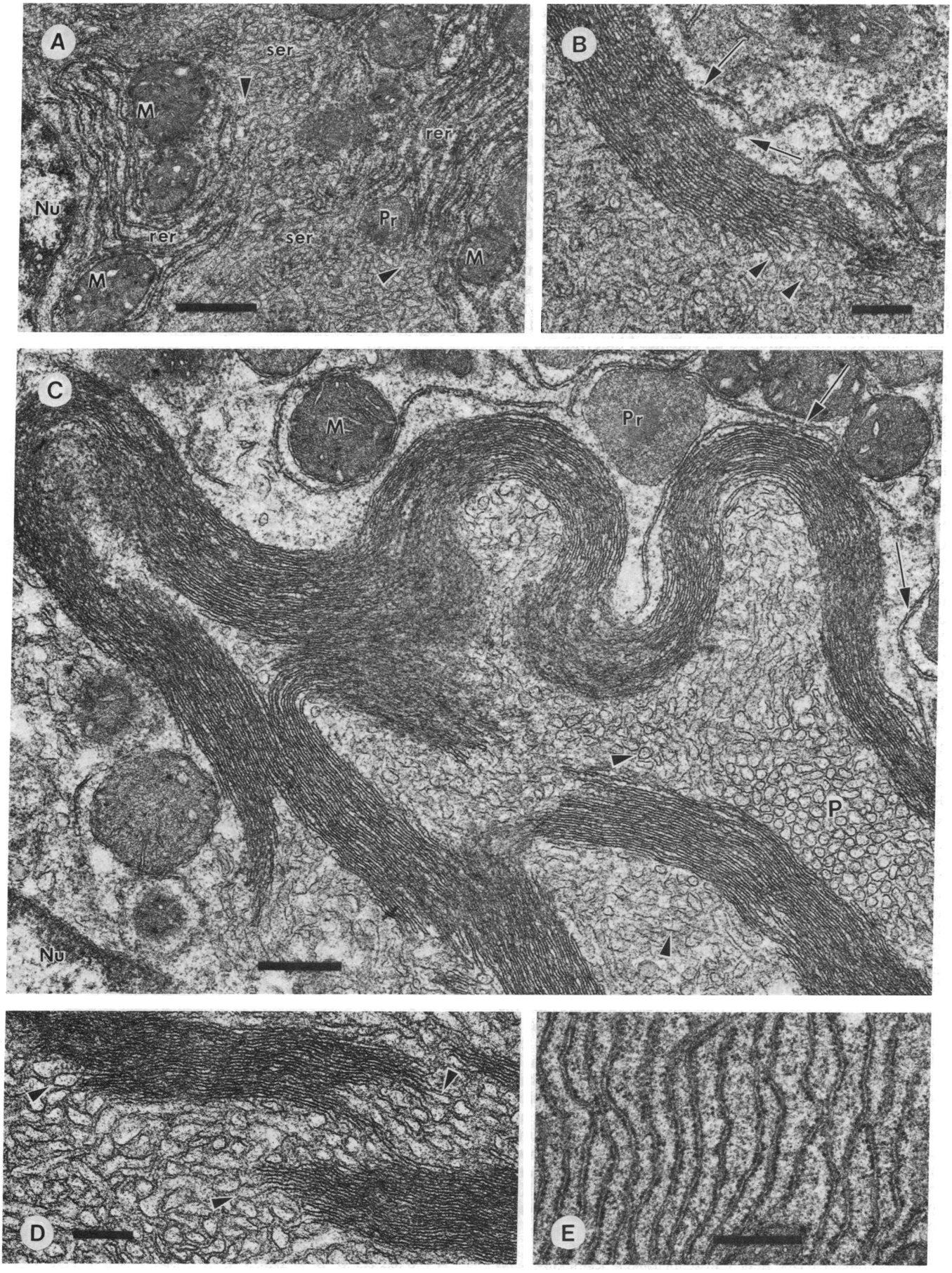

FIG. 4. (*Legend appears at the bottom of the preceding page.*)

cytes of normal control rats do not synthesize detectable amounts of HMG-CoA reductase, they may be induced to do so by various regimens of mevinolin and cholestyramine. These observations strongly suggest that the synthetic pathway for HMG-CoA reductase is markedly down-regulated in untreated rat livers. We initially thought that this scattered recruitment of hepatocytes might be due to sampling at the low point in the diurnal rhythm for HMG-CoA reductase synthesis (14). However, we found that although livers at the diurnal high point expressed 5-fold more enzyme activity, the labeling was still distributed periportally in a minority of the normal (untreated) hepatocytes. Therefore, HMG-CoA reductase appears to be synthesized at low levels in control rat livers. Stimulation of enzyme synthesis apparently results from relief of a dual negative feedback mechanism in which (*i*) the rate of liver cholesterol synthesis is increased due to the sequestering of intestinal bile acids by cholestyramine (30) and (*ii*) available HMG-CoA reductase is bound by mevinolin (16). In addition to an enhanced biosynthetic capacity (15–17), it is also likely that the increased numbers of reductase-positive hepatocytes and the stimulation of reductase activity that we observed are due to heightened enzyme stability (16). These extraordinary increases in hepatic HMG-CoA reductase activity may partially explain why mevinolin does not lower serum cholesterol levels in rats over long term periods (11, 12).

We have also demonstrated with EM that the SER is extensively developed in hepatocytes whose HMG-CoA reductase activity is greatly enhanced. The increase in mevinolin-treated rats is primarily in the form of randomly scattered masses of anastomosing SER tubules, with less conspicuous stacks of SER cysternae. The frequency of these SER stacks increases with the duration of mevinolin treatment, and they are most prominent in rats dosed with mevinolin and cholestyramine. In the latter case, both SER tubules and conspicuous whorles of tightly stacked SER cysternae are concentrated into massive aggregates localized near the nucleus. Since HMG-CoA reductase is an integral microsomal membrane protein (23), which has been localized on the SER membranes with immunoelectron microscopy (21), the juxtanuclear globules of reductase-specific stain in our IF preparations evidently correspond to the SER aggregates observed with EM. This SER proliferation is probably coupled, by some unknown mechanism, to the increased reductase synthesis induced by mevinolin. The points of continuity that we observed between the RER, stacks of SER cysternae, and SER tubules suggest that the SER is derived from the RER in a manner analogous to other systems (31). Similarly, a conspicuous SER is present in the steroidogenic cells of the adrenal cortex, the testis, the corpus luteum, and the lemur antebrachial organ (31, 32) and in UT1 cells grown in the presence of high levels of compactin (19, 20). The SER of all of these cell types is organized as extensive paracrystalline arrays of membranous SER tubules, in contrast to our system, in which the most prominent SER structures are the stacks of cysternae. This difference is possibly due to the inordinately high quantities of HMG-CoA reductase produced in response to treatment with mevinolin and cholestyramine and to physiological differences between rat hepatocytes and the other cell types. Phenobarbital administration also induces a hepatic SER proliferation in rats, consisting of masses of anastomosing SER tubules without conspicuous stacks of SER cysternae (33, 34). Taken together, these data suggest that mevinolin probably does not directly stimulate SER proliferation and perhaps that SER expansion occurs to provide a scaffolding for the vast increases of intramembranous proteins synthesized.

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