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Estrogen and Hydroxysteroid Sulfotransferases in Guinea Pig Adrenal Cortex: Cellular and Subcellular Distributions*

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ABSTRACT

This report describes for the first time the cellular and subcellular localization of estrogen sulfotransferase (EST) as well as the subcellular localization of hydroxysteroid sulfotransferase (HST) in the mammalian adrenal cortex. A 34-kilodalton EST and two HSTs with 3α- and 3β-hydroxysteroid substrate specificities (32 and 33 kilodaltons, respectively) were previously purified from guinea pig adrenal cortex and characterized. Western blots were used to establish that two antisera generated against EST were highly specific for EST, whereas three antisera generated against the HSTs were highly specific for the HSTs, but did not distinguish between the 3α- and 3βHSTs. Light and electron microscopic immunoperoxidase labeling with these antisera revealed that the sulfotransferases were expressed only within the ACTH-responsive layers of the guinea pig adrenal cortex, with EST localized to zona fasciculata and zona reticularis cells, and the HSTs confined to the zona reticularis. No labeling was detected in the zona glomerulosa or adrenal medulla. EST was concentrated in cell nuclei; sparse labeling was distributed throughout the cytoplasm. HST labeling was intense in smooth endoplasmic reticulum of zona reticularis cells, but was absent from nuclei. Ovoid inclusions about 1–4 μm in diameter, with no limiting membrane, were observed in zona reticularis cells; these inclusions were strongly labeled for both EST and HSTs. No gender-specific differences in distribution or labeling intensity were apparent. The high concentration of EST immunoreactivity in nuclei suggests that EST may play a role in modulating the ability of active estrogens to regulate gene expression in ACTH-responsive cells. The distribution of HST labeling suggests that sulfonation of adrenocortical 3-hydroxysteroids takes place largely within smooth endoplasmic reticulum in the zona reticularis in adult guinea pigs. (*Endocrinology* **133:** 2284–2291, 1993)

TISSUE concentrations of active steroid hormones are regulated in part by sulfotransferases (1–3). Sulfonation introduces an SO_3^- residue that remains fully ionized at the pH of biological systems (1), reducing the affinity of steroids for their intracellular receptors (4). However, sulfated steroids, such as pregnenolone sulfate and dehydroepiandrosterone sulfate (DHA SO_4), can have biological actions on their own (5–7) and can serve as substrates in enzymatic reactions (4). In addition, sulfonation of steroids increases water solubility and may increase transportability (1).

The adrenal cortex, after the liver, is perhaps the most active tissue for sulfonating steroids. One of the most abundant steroids produced by the human and primate adrenal cortex is DHA SO_4 (8, 9). DHA SO_4 can be converted to both androgens and estrogens in peripheral tissue (10). During human fetal development, DHA SO_4 produced in the fetal adrenal is ultimately converted into estrogens and accounts for 50% and 90% of maternal circulating estradiol and estriol, respectively (11). Two relatively uncharacterized adrenocortical steroid sulfotransferase activities have been studied for some time: hydroxysteroid sulfotransferase (HST) and estrogen sulfotransferase (EST) (4). We recently cloned and expressed cDNA for the guinea pig adrenocortical EST (12) and characterized the expressed enzyme (13). In addition, we have recently characterized two closely related HSTs with distinct substrate specificities (14): a 32-kilodalton (kDa) enzyme that sulfonates steroids with a 3α-hydroxyl group [allopregnanolone (3α-hydroxy-5α-pregnan-20-one) and androsterone] and a 33-kDa enzyme that is specific for 3β-hydroxysteroids (pregnenolone, 17-hydroxypregnenolone, and DHA).

To obtain evidence concerning the roles of the sulfotransferases in the regulation of adrenal steroidogenesis, we determined their cellular and subcellular distributions within the functionally distinct layers of the guinea pig adrenal cortex. The ACTH-responsive layers of the guinea pig adrenal cortex comprise the zona reticularis and zona fasciculata (15, 16). Although the guinea pig zonae reticularis and fasciculata both respond to ACTH with elevations of adenyl cyclase activity and cAMP levels, only the zona fasciculata increases cholesterol side-chain cleavage in response to ACTH and stress (17–19). The zona glomerulosa produces mineralocorticoids and is normally regulated by the renin-angiotensin system and potassium (15, 16). We present evidence here that EST is present in both nuclei and cytoplasm in zonae reticularis and fasciculata, and that HSTs are restricted to the zona reticularis and are highly concentrated in smooth endoplasmic reticulum, but absent from nuclei. Both types of sulfotransferase are concentrated in ovoid inclusions

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about 1–4 μm in diameter found only in zona reticularis cells.

Materials and Methods

Animals

Male and female guinea pigs, weighing 500–900 g, were used. NIH strain 2 guinea pigs were obtained from the NCI, and Hartley guinea pigs were obtained from Harlan Sprague-Dawley (Indianapolis, IN). They were maintained on guinea pig chow and water *ad libitum* on a controlled light-dark cycle at constant temperature and humidity.

Antibodies

The rabbit antisera against the 34-kDa EST and the 32- and 33-kDa HSTs were generated and characterized as described previously (12, 14, 20, 21). Antisera CS-455 and CS-456 were generated against purified EST (pI 6.4 and pI 5.2 isoforms, respectively). Antisera BD-442, BD-441, and CS-457 were prepared against purified 3αHST, 3βHST, and a previously uncharacterized protein we now know to be 3αHST by immunochemical criteria, respectively.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

Adrenocortical cytosol and 3′,5′-ADP agarose affinity-purified HSTs were prepared exactly as previously described (14). Proteins were resolved by SDS-PAGE in 15% polyacrylamide gels, as described by Laemmli (22), and visualized by silver staining without glutaraldehyde fixation (23). For immunochemical analysis, proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH), as described by Towbin *et al.* (24). Western blotting and colorimetric visualization of immunoreactive proteins were carried out exactly as described previously (20). All antisera were used at a dilution of 1:4000.

Light microscopic immunocytochemistry

Animals were anesthetized with isoflurane, the chest wall was opened, a cannula was inserted into the left ventricle, and a small incision made in the right atrium. Room temperature PBS, pH 7.4, was perfused through the animal by gravity flow for approximately 30 sec, after which the perfusion fluid was changed to 4% formaldehyde (freshly depolymerized paraformaldehyde), 0.2% picric acid, and 0.1 M sodium cacodylate, pH 6.0. Blocks of tissue were placed in fixative for 2 h; the tissues were cryoprotected in 10% sucrose in PBS and frozen on dry ice, and 12-μm sections were cut with a cryostat and picked up on gelatin-coated glass slides. Endogenous peroxidase activity was suppressed by treating sections for 5 min with 3% hydrogen peroxide and 10% methanol in PBS. Tissues were processed using Vector avidin-biotinylated peroxidase reagents (Vector Laboratories, Burlingame, CA), as described previously (21). All antisera were used at dilutions of 1:4000. Counterstaining and optical methods designed to increase the contrast of unstained tissue were not employed so as avoid obscuring the label.

Electron microscopic immunocytochemistry

Animals were perfused through the heart with 4% glutaraldehyde, 0.2% picric acid, and 0.1 M sodium cacodylate, pH 6.0. Adrenal gland slices were incubated in 1% osmium tetroxide for 1 h, rinsed in water, incubated in 70% ethanol for several hours, infiltrated with LR white overnight, and polymerized at 50 C. Ultrathin sections were picked up on Formvar-coated nickel slot grids and stained with the above antisera at dilutions of 1:400. Immunoperoxidase labeling was accomplished as described previously (25–27).

Conventional electron microscopy

Fixation of minced adrenal glands in buffered aldehydes, osmication, embedment in Epon, and contrast enhancement of ultrathin sections with lead and uranyl salts were performed using standard techniques.

Results

Antibody characterization

The specificities of the antibodies used in this study were analyzed by Western blot, and the results are illustrated in Fig. 1. Affinity-purified 3αHST and 3βHST (lane 2 of each pair in Fig. 1) were run in parallel with the cytosolic preparation (lane 1 of each pair). The CS-456 antiserum reacted against a single 34-kDa cytosolic protein (Fig. 1A) that is known to be EST (12). Antiserum CS-455 produced identical results (not shown). The BD-441, BD-442, and CS-457 antisera all reacted specifically with two cytosolic proteins of 33 and 32 kDa (Fig. 1, B, C, and D) that represent 3βHST and 3αHST, respectively (14). As shown in Fig. 1, the EST antiserum did not cross-react with HSTs, and the HST antisera did not cross-react with EST. As all three HST antisera reacted with both HSTs, no differential localization of 3αHST or 3βHST could be accomplished. Antisera CS-455 and CS-456 produced identical results in all immunochemical and immunocytochemical assays, as did antisera BD-441, BD-442, and CS-457.

Cellular sulfotransferase localization

At the light microscopic level, reaction product for EST was found in the zona fasciculata and zona reticularis, whereas the zona glomerulosa and adrenal medulla remained unstained (Fig. 2). In addition, nuclear staining was

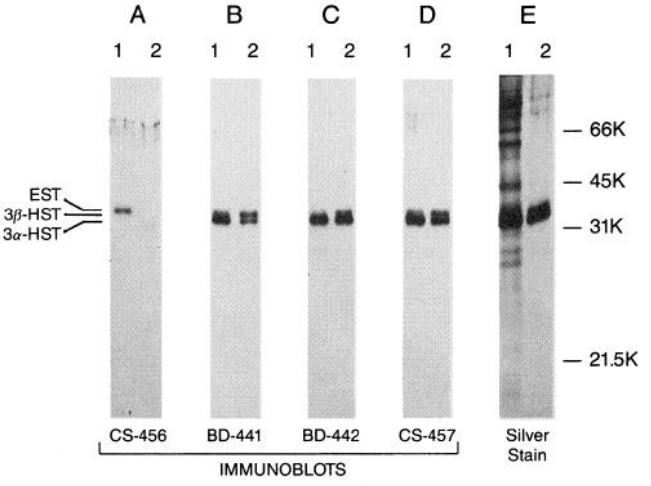

FIG. 1. Characterization of antisera by Western blot analysis. SDS-PAGE, silver staining, and immunoblotting were performed as described in *Materials and Methods*. For each immunoblot (A–D), the antiserum used is indicated. Lane 1 in each panel contained guinea pig adrenocortical cytosol (1.4 μg for A and E; 0.7 μg for B–D). Lane 2 in each panel contained 3′,5′-ADP agarose affinity-purified HSTs (0.42 μg for A; 0.14 μg for B–D; 0.28 μg for E). The identity of immunoreactive bands is indicated to the *left* of the immunoblots. The positions of mol wt markers are shown to the *right* of the silver-stained gel.

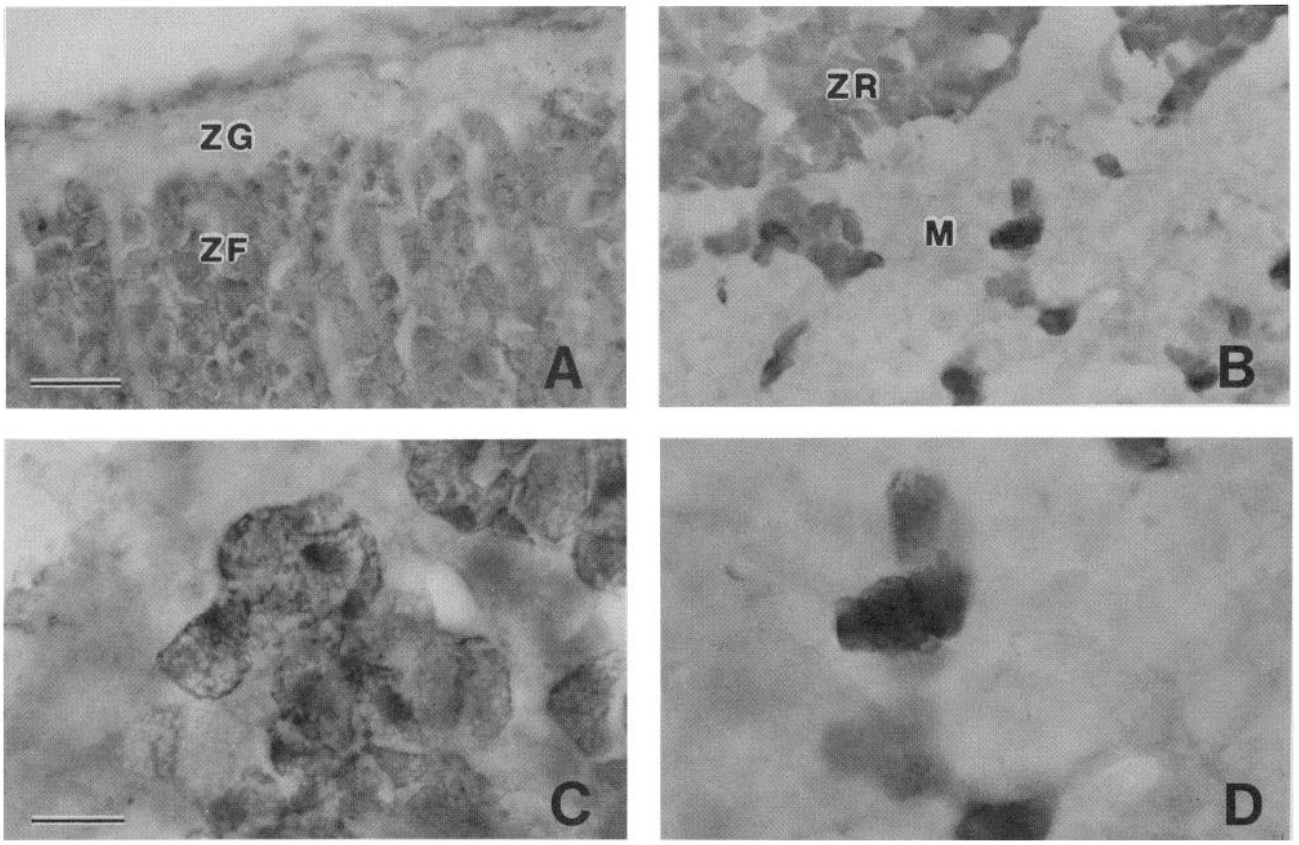

FIG. 2. Light microscopic (LM) distribution of EST (labeled with antiserum CS-455) in the zonae fasciculata (ZF) and reticularis (ZR) of a female Hartley guinea pig. A, Outer region of zona fasciculata bordering unlabeled zona glomerulosa (ZG). B, Inner region of zona reticularis bordering unlabeled adrenal medulla (M). C, Higher magnification of border between zona fasciculata and zona glomerulosa. D, Higher magnification of isolated zona reticularis cells in medulla. Original magnifications for A and B, ×212.5; calibration *bar* = 50 μm. Original magnifications for C and D, ×531.25; calibration *bar* = 20 μm.

observed; many cell nuclei appeared to be more intensely labeled than the surrounding cytoplasm (Fig. 2). No gender-specific differences in intensity or distribution of peroxidase reaction product were observed.

When cryostat sections were incubated with antisera against HSTs, staining was confined to the zona reticularis (Figs. 3 and 4). As for EST, no gender-specific differences were seen in intensity or distribution of labeling (Fig. 3). Reaction product was confined to intensely stained regions within cells, rather than being diffusely spread throughout cells as was observed with EST antisera (compare Fig. 4C to Fig. 2D). No obvious nuclear staining was present for the HSTs (Figs. 3 and 4).

Immunoreactivity for EST or HSTs was absent from the zona glomerulosa and adrenal medulla (Figs. 2–4) as well as the olfactory lobe, brain stem, liver, kidney, and testis (not shown). Incubation of cryostat sections with preimmune sera from the rabbits used to produce the EST and HST antisera resulted in no staining (shown for rabbit BD-441 in Fig. 4D).

Subcellular sulfotransferase localization

To identify the organelles responsible for the light microscopic pattern of labeling observed for HSTs and to confirm the nuclear labeling indicated for EST, we employed postembedding immunoperoxidase labeling of ultrathin LR white sections for electron microscopy. All antisera against EST or HSTs resulted in punctate labeling within specific subcellular regions of steroid-producing cells (Fig. 5, A–D). Similar to the results with cryostat sections, zona glomerulosa and medullary chromaffin cells remained unlabeled by these antisera (not shown). Incubation of ultrathin sections with preimmune sera from the rabbits used to produce the EST and HST antisera resulted in no staining (not shown). The HST antisera also failed to stain zona fasciculata cells with this technique (not shown), consistent with the light microscopic results (Figs. 3 and 4).

Clear differences were seen in the subcellular distributions of EST and HSTs (compare Fig. 5, A and B, to Fig. 5, C and D). EST antisera-labeled cell nuclei more intensely than cytoplasm, which generally displayed punctate staining spread throughout the cell. In the zona reticularis, but not the zona fasciculata, ovoid inclusions, 1–4 μm in diameter, were frequently observed in the cytoplasm and were stained as strongly for EST as the nucleus (Fig. 5B). These inclusions are referred to here as dense bodies. The dense bodies were identical in shape, size, and location to the large oval inclusions seen in zona reticularis cells by conventional electron

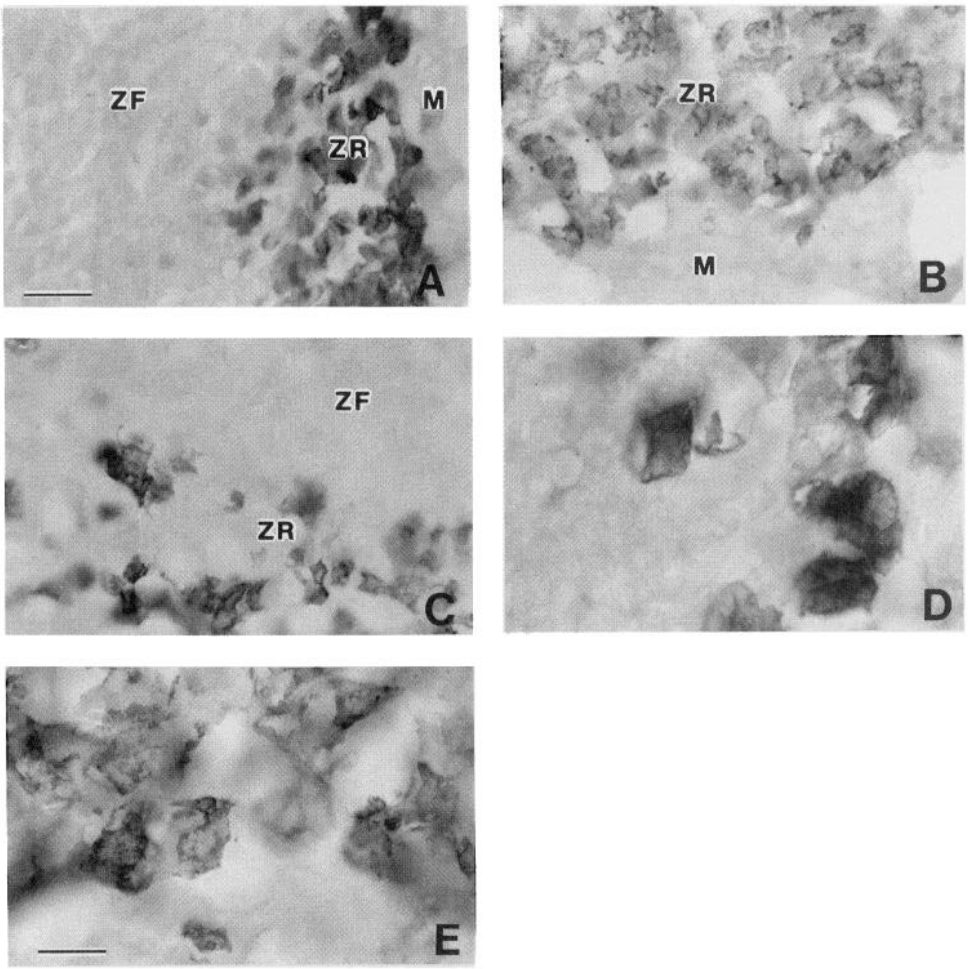

FIG. 3. LM distribution of HSTs in the zona reticularis (labeled with antiserum CS-457) of male and female NIH guinea pigs. A, Zona reticularis of a male bordered on either side by unlabeled medulla and unlabeled zona fasciculata. B, Inner zona reticularis of a female bordered by medulla. C, Outer zona reticularis of a female adjacent to zona fasciculata. D, Higher magnification of zona reticularis cells of a male, bordered by zona fasciculata. E, Higher magnification of zona reticularis cells of a female, adjacent to medulla. Abbreviations are explained in Fig. 1. Original magnifications for A, B and C, ×212.5; calibration *bar* = 50 μm. Original magnifications for D and E, ×531.25; calibration *bar* = 20 μm.

microscopy (Fig. 5E). These regions possessed a finely grained structure (Fig. 5, D and E) and were not limited by a membrane (Fig. 5E). EST antisera labeled stacks of parallel smooth endoplasmic reticulum with the same low intensity as the rest of the cytoplasm (not shown). The HST antisera, on the other hand, intensely labeled smooth endoplasmic reticulum stacks (Fig. 5C), but did not label nuclei (Fig. 5, C and D). Staining of the dense bodies with HST antisera was similar to results obtained with EST antisera (compare Fig. 5D with Fig. 5B). In addition, sparser punctate labeling was spread throughout the rest of the cytoplasm (Fig. 5D).

Discussion

This is the first report on the cellular distribution of EST as well as the subcellular distribution of both EST and HSTs in the mammalian adrenal cortex. The present study yielded essentially five main findings. 1) EST was present in the zona fasciculata and the zona reticularis of the guinea pig adrenal cortex, whereas HSTs (3αHST and 3βHST) were present only in the zona reticularis. 2) Neither EST nor HSTs were present in the zona glomerulosa or medulla. 3) EST was concentrated in cell nuclei, whereas HSTs were absent from the nucleus. 4) HST labeling was intense in stacks of smooth endoplasmic reticulum in zona reticularis cells, whereas EST antisera produced only sparse labeling in these organelles. 5) Large ovoid inclusions (dense bodies) in the cytoplasm of zona reticularis cells were strongly labeled for both EST and HSTs. Sulfotransferases are described in the literature as being soluble or cytosolic enzymes, with no reference to cellular structure. It is evident from the results reported here that adrenocortical steroid sulfotransferases are associated with specific organelles: EST with the nucleus and dense bodies, and HSTs with smooth endoplasmic reticulum and dense bodies, but not the nucleus.

The identity of the dense bodies that stained intensely for EST and HSTs in LR white ultrathin sections is unknown. They do not appear to result from break-up of smooth endoplasmic reticulum during fixation, as they do not contain vesicles (28). The dense bodies are not obliquely sectioned stacks of smooth endoplasmic reticulum, because the two organelles displayed strikingly different labeling patterns with EST antisera. These organelles may be related to lipid droplets, as lipid droplets can display a finely grained internal structure in zona reticularis in ultrathin sections (28), and lamellae are visible in some lipid droplets in freeze fracture (28).

Our finding of nuclear localization of EST is consistent with preliminary light microscopic evidence obtained in hepatocytes by others (29). As it is known that estrogen sulfo-

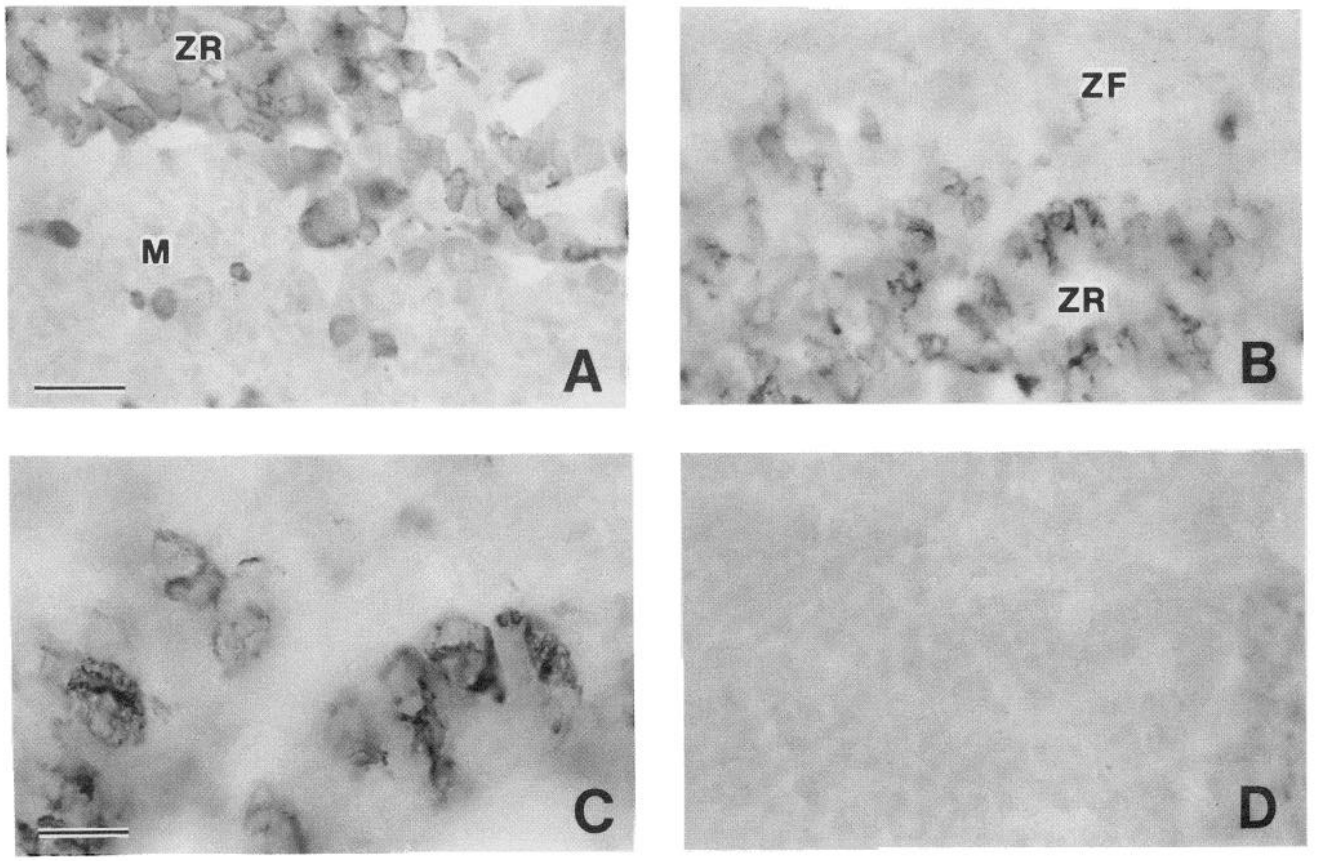

FIG. 4. LM distribution of HSTs in the zona reticularis of a male Hartley guinea pig labeled with antiserum BD-441. A, Inner region of zona reticularis bordering adrenal medulla. B, Outer zona reticularis bordered by zona fasciculata. C, Higher magnification of border between zona reticularis and zona fasciculata. D, Absence of labeling of zona reticularis with preimmune serum from rabbit BD-441. Abbreviations are explained in Fig. 1. Original magnifications for A, B, and D, ×212.5; calibration *bar* = 50 μm. Original magnification for C, ×531.25; calibration *bar* = 20 μm.

conjugates will not bind to the estrogen receptor, sulfoconjugation may be an important mechanism to regulate the concentration of the free, biologically active hormone (30, 31). Estrogen receptors are located in nuclei of adrenocortical cells in the monkey, and as reported here for adrenocortical EST, no gender-specific difference in immunocytochemical labeling was found for adrenocortical estrogen receptors (32). *In vitro* studies have shown direct effects of estrogens on adrenocortical steroidogenesis (33, 34). The nuclear localization of EST in steroid-producing cells of the zonae fasciculata and reticularis suggests that EST could affect gene regulation by estrogens in these cells.

EST may be involved in the processing of estrogens synthesized by the adrenal cortex. Although it is stated that little if any estrogen is synthesized by this tissue, we have found 3000–9000 fmol/g estradiol and estrone in guinea pig adrenal cortex; serum concentrations ranged from less than 30 to about 300 fmol/ml (unpublished observations). Estradiol sulfate and estrone sulfate are present in adrenocortical tissue in concentrations of approximately 2000–4000 fmol/g (our unpublished observations). Such data strongly support the notion that unconjugated and sulfonated estrogens are indeed produced in the guinea pig adrenal cortex. Nevertheless, although these findings are of interest, the biological significance of the estrogens and EST in the adrenal cortex and their influence on adrenocortical function must await further experimental developments.

Because the HST antisera did not distinguish between 3αHST and 3βHST on Western blots, we do not know whether labeling with the HST antisera represented localization of 3αHST, 3βHST, or both. In all likelihood, each HST antiserum stained both 3αHST and 3βHST with the immunocytochemical techniques used here as they did on the Western blots. The localization of 3βHST to the zona reticularis is consistent with previous reports localizing DHA sulfotransferase to this adrenocortical region (35–37) and with the fact that the 3β-hydroxysteroid sulfoconjugates of pregnenolone, 17-hydroxypregnenolone, and DHA have been measured in the guinea pig adrenal cortex, and in each case the concentration was significantly higher in the zona reticularis *vs.* the fasciculata/glomerulosa (38). On the other hand, localization of 3αHST has not been previously reported, so this communication represents the first indication that it also localizes to the zona reticularis. To our knowledge, concentrations of 3α-hydroxysteroid sulfoconjugates have not been measured in the adrenal cortex, but one would anticipate that they, too, would be highest in the zona reticularis. An interesting and unexpected observation is that 3αHST appears to be such an overall prominent protein in adrenal cytosol. Whereas it might seem unlikely that the

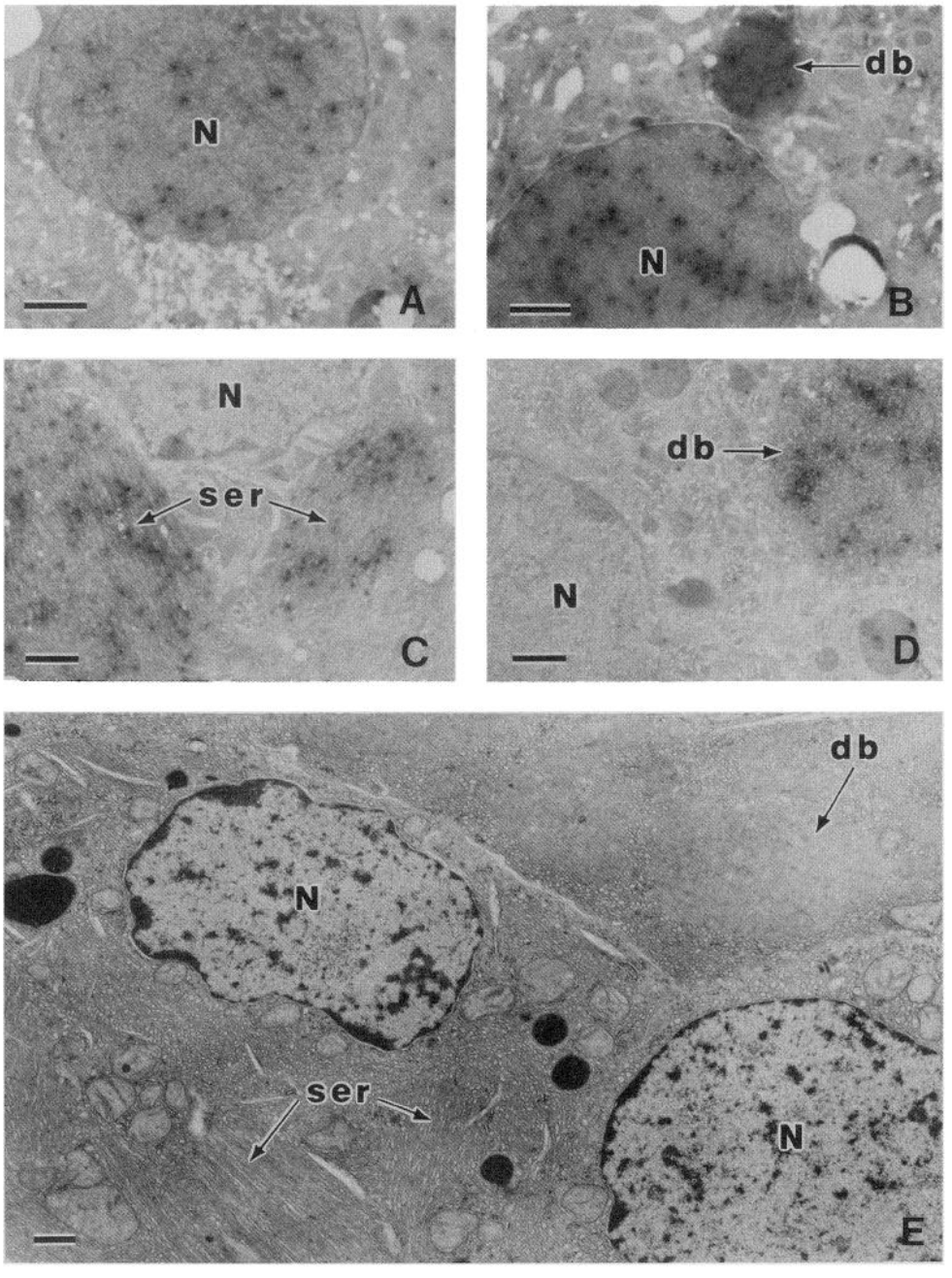

FIG. 5. Electron microscopic (EM) localization of EST and HSTs in steroid-producing cells of adrenal cortex. A, Nuclear (N) localization of EST in zona fasciculata cell (antiserum CS-455). B, Labeling of nucleus and dense body (db) in cytoplasm for EST in zona reticularis cell (antiserum CS-456). C, Labeling of parallel stacks of smooth endoplasmic reticulum (ser) for HSTs in zona reticularis cell (antiserum CS-457). D, Labeling of dense body for HSTs in zona reticularis cell (antiserum CS-457). E, Conventional EM of smooth endoplasmic reticulum and dense body in zona reticularis cells for comparison. Original magnifications for A and B, ×10,500. Original magnifications for C and D, ×8,970. Original magnification for E, ×7,000. Calibration *bars* = 1.0 μm.

entire broad 32-kDa band in adrenal cytosol is 3αHST, a quantitative estimate for the enzyme can be made based on the fact that the 3αHST antibody (Fig. 1C) reacted quantitatively with similar 32-kDa bands in adrenal cytosol (lane 1) and affinity-purified protein (lane 2). Using the amounts of applied protein (0.7 μg for lane 1 and 0.14 μg for lane 2) and an estimated 3αHST to 3βHST ratio of 2:1 for the affinity-purified preparation, then 3αHST constitutes a remarkable 13% of the total adrenal soluble protein. Furthermore, the amount of 3αHST overwhelms those of 3βHST and EST. The predominance of 3αHST in the reticularis suggests a role of some significance for this enzyme in this cell type.

The mammalian adrenal cortex is composed of essentially three concentric zones (15, 16): the outermost zona glomerulosa is the source of aldosterone and is regulated by the renin-angiotensin system and potassium; the middle zona fasciculata is the source of cortisol and is regulated by ACTH; and the inner-most zona reticularis is an enigma, for its exact function is not well understood. In the guinea pig, the zona reticularis is unusually large, and although reticularis cells contain functional ACTH receptors, there is no stimulation of side-chain cleavage activity in response to stress (17) and no stimulation of cortisol production in response to ACTH treatment (18), in contrast to zona fasciculata cells. In fact,

reticularis cells of the guinea pig adrenal cortex appear to have a defect in ACTH action at a step beyond the formation of cAMP (19). It is intriguing that specific steroid sulfotransferases are differentially expressed in cells of functionally distinct adrenocortical zones; however, a meaningful relationship remains to be determined. In the guinea pig model, certain questions can be raised, such as, why is EST so abundant in fasciculata and reticularis cells? Does EST function solely as an estrogen sulfotransferase or might it have another role? This possibility is raised by the finding that the ratio of unconjugated to sulfoconjugated estrogens in the adrenal cortex is approximately 2:1 (our unpublished findings). For that matter, what function(s) might the estrogens influence? As it is now clear that sulfonation of nonestrogenic 3-hydroxysteroids can occur only within the zona reticularis, what is the meaning of this restriction? Substrates for the HSTs are presumably present in both the zona fasciculata and the zona reticularis. Another intriguing question is why are EST and HSTs absent from the zona glomerulosa? It is anticipated that when the cDNAs and genes for the individual members of the steroid sulfotransferase family are cloned, answers to such questions can be more directly determined.

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