Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 1458–1462, February 1992
Biochemistry

Role of steroid 11β-hydroxylase and steroid 18-hydroxylase in the biosynthesis of glucocorticoids and mineralocorticoids in humans

(cortisol synthesis/aldosterone synthesis/corticosterone methyl oxidase/P-450 enzymes)

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Communicated by Seymour Lieberman, November 7, 1991 (received for review June 29, 1991)

ABSTRACT **A gene encoding steroid 18-hydroxylase (P-450$_{C18}$) was isolated from a human genomic DNA library. It was identified as *CYP11B2*, which was previously postulated to be a pseudogene or a less active gene closely related to *CYP11B1*, the gene encoding steroid 11β-hydroxylase (P-450$_{11\beta}$) [Mornet, E., Dupont, J., Vitek, A. & White, P. C. (1989) *J. Biol. Chem.* 264, 20961–20967]. The nucleotide sequence of the promoter region of the P-450$_{C18}$ gene is strikingly different from that of the P-450$_{11\beta}$ gene, although the sequences of their exons are 93% identical. The transient expression in Y-1 adrenal tumor cells of CAT constructs with a series of deletion mutants of promoter regions of both genes indicated that the two genes are regulated differently. P-450$_{C18}$ as expressed in COS-7 cells exhibits steroid 18-hydroxylase activity to catalyze the synthesis of aldosterone and 18-oxocortisol and exhibits steroid 11β-hydroxylase activity as well. In contrast, P-450$_{11\beta}$ as expressed in the cultured cells exhibits steroid 11β-hydroxylase activity exclusively but fails to catalyze the synthesis of aldosterone and 18-oxocortisol. These results indicate that P-450$_{11\beta}$ and P-450$_{C18}$ are products of two different genes and that the former participates in the synthesis of glucocorticoids whereas the latter participates in the synthesis of mineralocorticoids in humans.**

Aldosterone, the most potent mineralocorticoid, is synthesized via enzymatic reactions involving several specific monooxygenases (1), termed P-450 (2), starting from cholesterol and proceeding through a pathway including pregnenolone, progesterone, 11-deoxycorticosterone (DOC), and corticosterone as metabolic intermediates (3). The molecular nature of the enzyme that catalyzes the final step of aldosterone biosynthesis is not fully understood. It has been postulated that steroid 11β-hydroxylase (P-450$_{11\beta}$), a product of *CYP11B* (4), is the enzyme responsible for the biosynthesis of aldosterone (5–7), because P-450$_{11\beta}$ purified from bovine adrenocortical mitochondria catalyzes the conversion of corticosterone to aldosterone via 18-hydroxycorticosterone (5), in addition to its main function, catalysis of the 11β-hydroxylation of DOC to form corticosterone. In contrast, Ogishima *et al.* (8) and Lauber and Müller (9) recently isolated aldosterone synthase cytochrome P-450 (P-450$_{aldo}$), which is induced in Na^+-depleted K^+-replete rat adrenal cortex (10, 11), and demonstrated that rat P-450$_{aldo}$ catalyzes three successive monooxygenation reactions of DOC to form aldosterone as a final product, whereas rat P-450$_{11\beta}$ does not substantially catalyze the reaction to form aldosterone.

In regard to aldosterone biosynthesis in the human adrenal cortex, it has been postulated for a long time (12–14) that two types of enzymes, corticosterone methyl oxidases type I (CMO I) and type II (CMO II), are involved in the final two steps of aldosterone synthesis, because several acquired and inborn errors in the synthesis or action of aldosterone (e.g., CMO II deficiency: hypoaldosteronism with elevated excretion of 18-hydroxycorticosterone and elevated level of plasma renin activity) cannot be explained by functional anomaly of P-450$_{11\beta}$ (13). Nevertheless, such CMOs have not been isolated.

Recently, we reported the isolation of two cDNAs coding for human P-450$_{11\beta}$ (15) and human steroid 18-hydroxylase [P-450$_{C18}$ (16); formerly designated P-450$_{aldo}$ (17)] and presented evidence that the P-450$_{C18}$ cDNA corresponds to the transcript of *CYP11B2*, which was postulated by Mornet *et al.* (18) to be a pseudogene or a closely related less active gene for P-450$_{11\beta}$. Subsequently, Ogishima *et al.* (20) isolated human P-450$_{C18}$ (or P-450$_{aldo}$) from aldosterone-producing adenoma and reported that their specific polyclonal antibody raised against a partial amino acid sequence deduced from a portion of presumed exons 1 and 2 of *CYP11B2* (18) does not react with human P-450$_{11\beta}$ but reacts with human P-450$_{C18}$ as judged by Western blot analysis, indicating that P-450$_{C18}$ is a product of *CYP11B2*. Here we present molecular genetic evidence that P-450$_{11\beta}$ and P-450$_{C18}$ are products of two different genes, *CYP11B1* and *CYP11B2*, respectively, and that P-450$_{11\beta}$ participates in the synthesis of glucocorticoids whereas P-450$_{C18}$ participates in the synthesis of mineralocorticoids in humans.‖

MATERIALS AND METHODS

Materials. Anti-bovine P-450$_{11\beta}$ IgG (19, 20) was a generous gift from F. Mitani and Y. Ishimura (Keio University). 18-Hydroxycortisol and 18-oxocortisol were prepared as described (21). [^{3}H]DOC (30.4 Ci/mmol; 1 Ci = 37 GBq), [^{3}H]corticosterone (84 Ci/mmol), and ^{125}I-labeled protein A (35 Ci/mg) were from Amersham. [^{3}H]Deoxycortisol (46.7 Ci/mmol), [^{3}H]cortisol (52.1 Ci/mmol), and [^{14}C]chloramphenicol (54.9 mCi/mmol) were from NEN.

Molecular Cloning and Nucleotide Sequencing of P-450$_{11\beta}$ and P-450$_{C18}$ Genes. A genomic DNA clone (gH11B1) with an ≈40-kilobase (kb) insert containing the P-450$_{11\beta}$ gene was isolated from a cosmid Lorist 2 library (15). A genomic DNA clone (gH11B2) with an ≈20-kb insert containing the P-450$_{C18}$

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Abbreviations: P-450$_{11\beta}$, steroid 11β-hydroxylase; P-450$_{C18}$, steroid 18-hydroxylase; P-450$_{aldo}$, aldosterone synthase cytochrome P-450; CMO, corticosterone methyl oxidase; CAT, chloramphenicol acetyltransferase; DOC, 11-deoxycorticosterone.

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‖The sequences reported in this paper have been deposited in the GenBank data base (accession nos. D90428 and D90429).

gene was isolated from a human Charon 4A genomic DNA library (ref. 22; supplied by the Japanese Cancer Research Resources Bank) by using the same probe as that used for cloning P-450$_{C18}$ cDNA (16, 17). The nucleotide sequence was determined by the dideoxy method (23).

Chloramphenicol Acetyltransferase (CAT) Assay. The 5′ flanking region of the P-450$_{C18}$ or P-450$_{11\beta}$ gene was inserted into the *Hin*dIII site of pSV00CAT (24). DNA transfection and CAT assay were as described (15, 17).

Analysis of Steroid Hydroxylase Activity of P-450s Expressed in COS-7 Cells After Transfection. Expression plasmids containing P-450$_{11\beta}$ cDNA (pSV11β) and P-450$_{C18}$ cDNA (pSVC18) were constructed as described (17). DNA was transfected by electroporation (25). To overcome the vari-

FIG. 1. Nucleotide sequence of the 5′ flanking region and a part of exon 1 of the human P-450$_{C18}$ gene (upper sequence) compared with that of the human P-450$_{11\beta}$ gene (lower sequence). The sequences were maximally matched by insertion of gaps (-). Numbers at right refer to nucleotide positions relative to the transcriptional initiation site at +1 (15–18). A "TATA box" is indicated by double underlining. A putative AP-1 element is shown by a broken underline. Putative AP-2 and cAMP-responsive elements are marked by thick and thin underlines, respectively. Long dashed arrows show a remarkably long palindromic sequence. Stars indicate identical nucleotides.

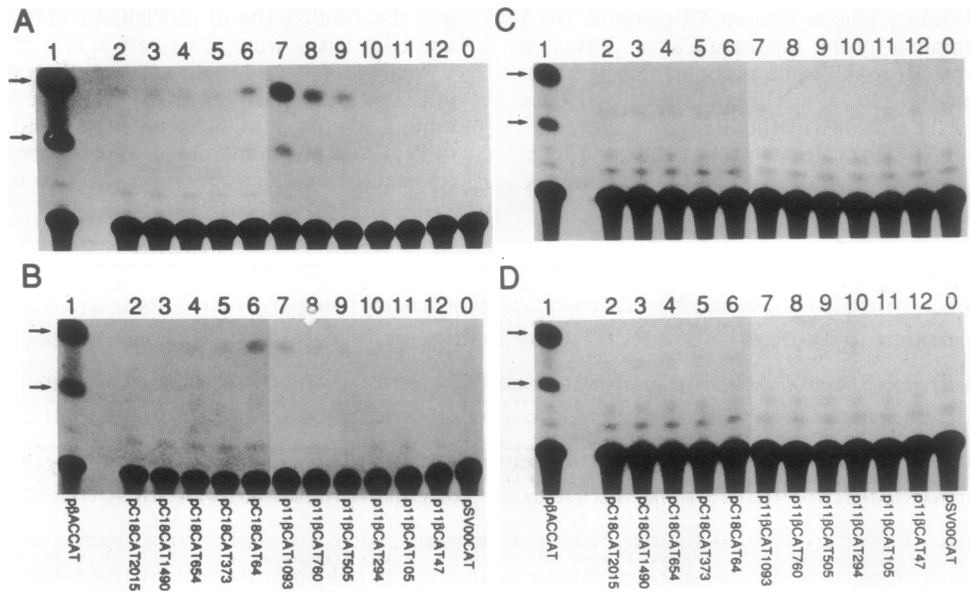

FIG. 2. Analysis of promoter activity of human P-450$_{C18}$ and P-450$_{11\beta}$ genes by CAT assay. A series of CAT chimeric constructs were transfected into Y-1 cells (*A* and *B*), BeWo cells (*C*), and HeLa cells (*D*). Cells were incubated in the presence (*A*, *C*, and *D*) or absence (*B*) of 1 mM 8-bromo cAMP. Lanes: 1, a human β-actin CAT construct [pβACCAT (30)] as a positive control; 2, pC18CAT2015, containing the 5′ flanking region of the P-450$_{C18}$ gene up to −2015; 3, to −1490; 4, to −654; 5, to −373; 6, to −64; 7, p11βCAT1093, containing the 5′-flanking region of the P-450$_{11\beta}$ gene up to −1093; 8, to −764; 9, to −505; 10, to −294; 11, to −105; 12, to −47; 0, pSV00CAT as a negative control. Arrows indicate acetylated chloramphenicol products.

ability inherent in transfection, pCH110 plasmid (26), which contains the *lacZ* gene, was cotransfected into COS-7 cells. The cells transfected were plated in 12 dishes (diameter, 90 mm) and incubated for 72 hr. The mitochondrial fraction was prepared (17), and protein concentration was determined (27). To check the purity of the mitochondrial fraction, succinate dehydrogenase activity was determined (28). To quantitate the amounts of P-450$_{11\beta}$ and P-450$_{C18}$ expressed in mitochondria of COS-7 cells, Western blot analysis (29) was performed using anti-bovine P-450$_{11\beta}$ IgG and ^{125}I-labeled protein A. Steroid hydroxylase activity was measured (17) using ≈1 mg of solubilized mitochondria. Radioactive products were analyzed (5) by reverse-phase HPLC on a Wakosil 5C$_{18}$ column (4.6 × 250 mm) with a mobile phase of aqueous 60% methanol at a flow rate of 0.3 ml/min.

RESULTS

In the first experiment, we attempted to isolate P-450$_{11\beta}$ and P-450$_{C18}$ genes from two types of human genomic DNA libraries in order to examine whether the two genes were identical with *CYP11B1* and *CYP11B2* (18), respectively. Two types of genomic clones, gH11B1 and gH11B2, were isolated. Nucleotide sequence analysis has revealed that the sequences of 9 exons in the P-450$_{11\beta}$ and P-450$_{C18}$ genes are 93% identical with each other and they coincide well with those of *CYP11B1* and *CYP11B2* (18), respectively, thus establishing that P-450$_{11\beta}$ and P-450$_{C18}$ are encoded by *CYP11B1* and *CYP11B2*. Since the promoter regions of *CYP11B1* and *CYP11B2* were not analyzed previously (18), we further determined the nucleotide sequences of the 5′ flanking regions of both genes (Fig. 1). The 5′ flanking region of the P-450$_{C18}$ gene contains a TATA box, three putative cAMP response elements, and two putative AP-2 elements, whereas that of the P-450$_{11\beta}$ gene contains a TATA box, a single putative cAMP response element, and two putative AP-1 elements. A remarkably long palindromic sequence is present in the upstream region of the P-450$_{C18}$ gene (−1734 to −1001). The 5′ flanking regions of the two genes are only 48% identical (see Fig. 1), in contrast to the strikingly high identity of all exons of both genes.

To further characterize these genes, various lengths of the 5′ flanking region of each gene were ligated to the bacterial CAT gene and their promoter activities were tested by transient-expression assay in Y-1 adrenal tumor cells (Fig. 2 *A* and *B*). The CAT construct containing the 5′ flanking region of the P-450$_{11\beta}$ gene up to −1093 exhibited a relatively strong promoter activity in the presence of 8-bromo cAMP. Deletion to −760 or −505 resulted in a considerable loss of promoter activity. Deletion to −294 caused a substantial loss of promoter activity, so that CAT activity was detectable only by prolonged autoradiography. Further deletion to −105 or −47 abolished promoter activity. In contrast, the CAT construct containing the 5′ flanking region of the P-450$_{C18}$ gene up to −2015 did not exhibit any promoter activity. When the region was deleted to −1490, −654, or −373, no promoter activity was detected. Nevertheless, a weak but apparent basal promoter activity was detected in Y-1 cells when the 5′ flanking region was deleted to −64. This indicates that the region between −2015 and −65 interferes with promoter activity of the P-450$_{C18}$ gene in Y-1 cells. Comparison of Fig. 2 *A* and *B* reveals that 8-bromo cAMP remarkably stimulates promoter activity of the various lengths of the 5′ flanking region of the P-450$_{11\beta}$ gene, whereas it has no effect on the P-450$_{C18}$ gene in Y-1 cells. Promoter activity of the various lengths of the 5′ flanking regions of both genes was further

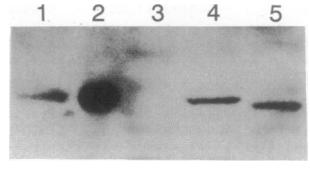

FIG. 3. Western blot analysis of P-450$_{11\beta}$ and P-450$_{C18}$ expressed in COS-7 cells. Lanes: 1, 12 ng of purified bovine P-450$_{11\beta}$; 2, 10 μg of bovine adrenocortical mitochondria; 3, 100 μg of mitochondria of COS-7 cells transfected with pSVL without insert; 4, 100 μg of mitochondria of COS-7 cells transfected with pSV11β; 5, 100 μg of mitochondria of COS-7 cells transfected with pSVC18.

Table 1. Steroid hydroxylase activity of mitochondria in COS-7 cells transfected with pSV11β or pSVC18

Substrate	Products	Retention time, min	Hydroxylase activity, pmol/mg of protein	
			pSV11β	pSVC18
DOC	Corticosterone	52	482.5 ± 19.0	437.9 ± 13.8
	18-Hydroxycorticosterone	29	2.7 ± 0.6	14.1 ± 4.3
	Aldosterone	24	<0.02	2.0 ± 0.5
11-Deoxycortisol	Cortisol	33	411.4 ± 23.3	392.5 ± 18.5
	18-Hydroxycortisol	21	1.1 ± 0.1	7.0 ± 0.7
	18-Oxocortisol	18	<0.02	1.8 ± 0.1
Corticosterone	18-Hydroxycorticosterone	29	1.5 ± 0.3	10.6 ± 2.8
	Aldosterone	24	<0.02	0.9 ± 0.2
Cortisol	18-Hydroxycortisol	21	0.7 ± 0.2	4.0 ± 0.8
	18-Oxocortisol	18	<0.02	0.4 ± 0.1

Hydroxylase activity of P-450s expressed in COS-7 cells was measured as described under *Materials and Methods*. Values represent mean ± SD of triplicate experiments for determining the amount of each product formed in a 20-min incubation with [^{3}H]DOC, [^{3}H]deoxycortisol, [^{3}H]corticosterone, or [^{3}H]cortisol as a substrate. Amounts of aldosterone and 18-oxocortisol formed from pSV11β (<0.02 pmol/mg) were below the limit of detection and represent the blank value obtained in mock transfection experiments. Amounts of 18-oxocortisol were determined on the basis of its purity as analyzed by periodic acid oxidation to form an etiolactone and a 17-ketosteroid (31).

examined with or without 8-bromo cAMP in steroidogenic BeWo choriocarcinoma cells and in nonsteroidogenic HeLa cells, but no promoter activity was detected using any of the deletion mutants (Fig. 2 *C* and *D*). These results indicate that the two genes are regulated differently and that their expression might be highly specific in the cells derived from the adrenal cortex.

To definitively clarify the functional characteristics of the products encoded by the P-450$_{11\beta}$ gene and the P-450$_{C18}$ gene, expression plasmids (pSV11β and pSVC18) with the 33 differences per 503 amino acid residues in both coding regions (16, 17) were transfected into COS-7 cells, and steroid hydroxylase activity and the amounts of these two P-450s expressed were determined. The amounts of P-450$_{11\beta}$ (M_r 50,000) and P-450$_{C18}$ (M_r 48,500) expressed in mitochondria of COS-7 cells were essentially the same as judged by Western blot analysis (Fig. 3). The 11β-hydroxylation of DOC or deoxycortisol to form corticosterone or cortisol catalyzed by these two P-450s proceeded to a similar extent (Table 1). No detectable amounts of the 18-oxo products, aldosterone and 18-oxocortisol, were formed in the presence of P-450$_{11\beta}$. In contrast, P-450$_{C18}$ catalyzed the formation of the 18-oxo products. In addition, P-450$_{C18}$ catalyzed the formation of the 18-hydroxy derivatives of corticosterone and cortisol to a much greater extent than P-450$_{11\beta}$. On the basis of these findings, Fig. 4 summarizes the metabolic pathways and enzymes involved in the biosynthesis of glucocorticoids and mineralocorticoids in humans. P-450$_{11\beta}$ catalyzes mainly reaction step 5, and P-450$_{C18}$ catalyzes steps 6 and 7 in addition to step 5.

DISCUSSION

We have isolated and characterized two genes for the biosynthesis of corticoids, one coding for P-450$_{11\beta}$ and the other coding for P-450$_{C18}$. At first glance, Table 1 might be interpreted to indicate that P-450$_{C18}$ plays a major role in the synthesis of both glucocorticoids and mineralocorticoids *in vivo* as well as *in vitro*. However, cortisol synthesis occurs predominantly in the zona fasciculata of the adrenal cortex and is controlled by corticotropin, whereas aldosterone is synthesized exclusively in the zona glomerulosa under the control of the renin–angiotensin system. The rate of aldosterone secretion is normally about 0.1% that of cortisol. Hence,

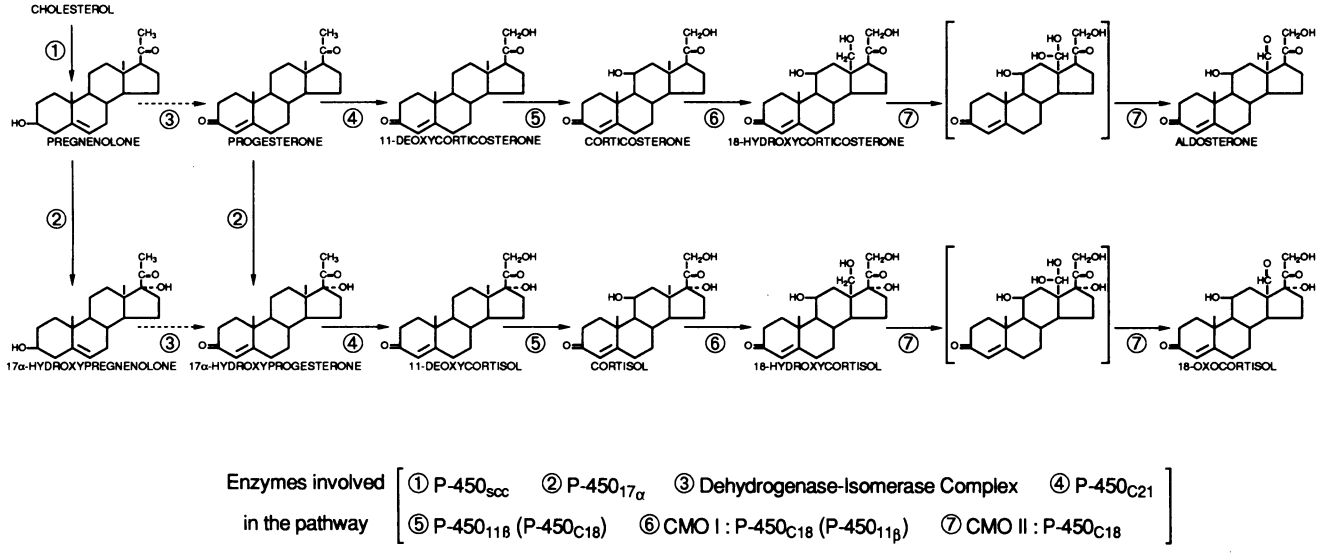

FIG. 4. Metabolic pathway and enzymes involved in biosynthesis of glucocorticoids and mineralocorticoids in humans. P-450$_{11\beta}$ catalyzes mainly reaction step 5, and P-450$_{C18}$ catalyzes steps 6 and 7 in addition to step 5.

the total amount of $P\text{-}450_{C18}$ expressed *in vivo* might be much lower than that of $P\text{-}450_{11\beta}$. Therefore, it is reasonable to conclude that glucocorticoids are synthesized *in vivo* mainly by $P\text{-}450_{11\beta}$. On the other hand, $P\text{-}450_{11\beta}$ does not substantially catalyze the formation of aldosterone (Table 1), but $P\text{-}450_{C18}$ catalyzes the synthesis of aldosterone, the most potent mineralocorticoid in humans. Thus, the major physiological function of $P\text{-}450_{C18}$ involves the synthesis of mineralocorticoids. Our current interpretation (Fig. 4) is that 18-hydroxylase activities of $P\text{-}450_{C18}$ involved in steps 6 and 7 correspond to CMO I activity and CMO II activity, respectively. A detailed molecular genetic analysis of CMO I and CMO II deficiencies should provide a definitive answer to the above interpretation.

In regard to nomenclature of P-450s in steroidogenesis, we emphasize that the term "$P\text{-}450_{C18}$" should be used in place of the term "$P\text{-}450_{aldo}$" as previously proposed (16). The term $P\text{-}450_{aldo}$ was originally used by Ogishima *et al.* (8) for the P-450 catalyzing the formation of aldosterone in rat, in which the cortisol synthetic pathway (see Fig. 4) is not operative due to lack of steroid 17α-hydroxylase. Further, the suffix letters (SCC, C21, 17α, 11β, and AROM) employed for the other five P-450s involved in the biosynthesis of steroids represent the enzymatic reactions catalyzed by these P-450s and they do not represent the reaction products formed. In other words, each suffix indicates which carbon atom or which moiety of steroids is modified by each P-450. In the case of the enzyme under discussion here, its main feature and physiological function lie in its ability to catalyze 18-hydroxylation of corticosterone and cortisol to finally produce aldosterone and 18-oxocortisol. Therefore, the term $P\text{-}450_{C18}$ is more comprehensive and suitable than the term $P\text{-}450_{aldo}$ for a better understanding of the biochemical features of this enzyme.

Note Added in Proof. After this article was accepted Curnow *et al.* (32) reported that *CYP11B2* is the gene for the aldosterone-synthesizing enzyme and that expression of the gene is regulated by angiotensin II. Therefore, these observations strongly support our previous (16, 17, 33) and current conclusion.

This work was supported in part by grants from the Uehara Memorial Foundation, Inamori Foundation, Takeda Science Foundation, Chiyoda Mutual Life Foundation, Toray Science Foundation, and Special Research Fund of Kochi Medical School and by Grants-in-Aid for Science and Cancer Research from the Ministry of Education, Science, and Culture of Japan.

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