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Studies on Cytogenesis in Adult Rat Adrenal Cortex: Circadian and Zonal Variations and Their Modulation by Adrenocorticotropic Hormone[1]

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Circadian rhythms and zonal variations in the cell proliferation of adult rat adrenal cortex were studied by following the cells in the DNA-synthesizing stage (S-phase) as assessed by 5-bromo-2′-deoxyuridine incorporation into the cell-nuclei and/or by visualizing proliferating cell nuclear antigen. The S-phase cells were observed throughout the day in two regions of the adrenal cortex: (i) a region from the inner half of the zona glomerulosa to near the outer margin of the zona fasciculata, and (ii) the outer one-fourth portion of the zona fasciculata. Very little change in number was observed in the former region between day and night, while a burst of cell proliferation occurred in early morning at 3-4 a.m. in the latter region. A prominent rise in the plasma adrenocorticotropic hormone (ACTH) concentration preceded the burst of cell proliferation by about 4 h. Upon raising the plasma ACTH concentration by administration of ACTH or metyrapone, prominent cell proliferation also occurred in the same portion of the zona fasciculata 4-6 h after the provoked ACTH surge. Thus at least two sites in rat adrenal cortex are responsible for cytogenesis in this endocrine organ, and respond differentially to day/night cycles and circulating ACTH levels.

Key words: adrenal cortex, adrenocorticotropic hormone (ACTH), circadian rhythm, proliferation.

Proliferation of cells in the adrenal cortex has been shown to exhibit a circadian rhythm through observation of such indices as mitotic division (*1-5*), nuclear volume (*2, 4*), and incorporation of ^{3}H-thymidine (*3, 6*). Some researchers reported that the circadian rhythm found in the mitotic index of both mice and rats had a proliferative peak during the daytime (*2-5*) or in early night (*1*), while others indicated that the rhythm observed in the nuclear volume or incorporation of ^{3}H-thymidine had a peak in the late night (*2, 4*) or in the morning (*3, 6*). Despite these differences in peak hours, which may reflect variations in the experimental conditions and techniques and/or differences in the animal strains used, all the investigators agreed that a circadian rhythm operated in the cell proliferation of the adrenal cortex (*1-6*).

We previously reported the presence of a cell layer without aldosterone synthase cytochrome P450 (P450aldo) and glucocorticoid-synthesizing enzyme (deoxycorticosterone 11β-hydroxylase cytochrome P450; $P450_{11\beta}$) between the zona glomerulosa and the zona fasciculata of the rat adrenal cortex (*7, 8*). The former enzyme catalyzes the synthesis of aldosterone from deoxycorticosterone (*9, 10*), while the latter produces corticosterone from the same substrate (*11, 12*). Similar cell layers that were devoid of both enzymes were also found in other rodents, such as mice (Mitani, F. *et al.*, unpublished observation). Its location and the properties of its constituent cells suggest that this layer may correspond to the cell region that previous investigators (*13-20*) termed "zona intermedia," "transitional zone," or "Sudanophobe zone," on the basis of morphological and/or histochemical observations made mostly before 1960. Unfortunately, however, the findings at that time provided little information on the function of this region. On the other hand, our finding that the cell layer is devoid of both P450aldo and $P450_{11\beta}$ indicates that the cells there are not able to produce glucocorticoids, mineralocorticoids, or adrenal androgens in significant quantities, and are thus functionally undifferentiated or immature: the function of the adrenal cortex is to produce corticosteroids. Furthermore, a relatively large number of replicating cells were found in and around the cell-layer, suggesting that the

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Abbreviations: ACTH, adrenocorticotropic hormone; Ad4BP/SF-1, Ad4-binding protein/steroidogenic factor-1; BrdU, 5-bromo-2′-deoxyuridine; FGF, fibroblast growth factor; IGF, insulin-like growth factor; P450aldo, aldosterone synthase cytochrome P450; $P450_{11\beta}$, deoxycorticosterone 11β-hydroxylase cytochrome P450; P450scc, cholesterol side-chain cleavage enzyme cytochrome P450; PCNA, proliferating cell nuclear antigen; RIA, radioimmunoassay; zF, the zona fasciculata; zFR, the zonae fasciculata-reticularis; zG, the zona glomerulosa; zR, the zona reticularis; zU, the undifferentiated cell zone of the adrenal cortex.

layer is the cell compartment providing new cells to the adrenal cortex. Since the cells in this layer are positive in both transcription factor Ad4BP/SF-1 (*21, 22*) and cholesterol side-chain cleavage enzyme cytochrome P450 (P450scc) (*23*), they are adrenocortical parenchymal cells and not of any other kind (*24*). Hence, we tentatively call this cell-layer the functionally undifferentiated cell zone of the adrenal cortex or simply the undifferentiated zone (zU) hereafter in this paper.

In an attempt to understand role(s) of the cells in the zU, we examined the temporal and spatial distributions of S-phase cells in and around the zU, and the effects of plasma adrenocorticotropic hormone (ACTH) concentration on the cell proliferation. The results revealed that two distinct sites function for cell proliferation, and that their sensitivities toward day/night cycle and ACTH were different. Based on these findings, previous hypotheses for the cell renewal and maintenance of the adrenal cortex in rats were reexamined.

MATERIALS AND METHODS

Animals—Male Sprague Dawley rats at 5 wk of age were obtained from Sankyo Labo Service Corporation, Tokyo. All animals were treated in accordance with the institutional animal care guidelines of the School of Medicine, Keio University. Before the experiments, rats were kept in our animal facility with a normal synthetic diet (*7*) for at least 1 wk. The animals were housed under a 12 h light (6 a.m.-6 p.m.)/12 h dark (6 p.m.-6 a.m.) cycle at 22±2°C under stable humidity with food and tap water available *ad libitum*. The rats were gently touched every day by one of the authors (H.M.) to minimize the unnecessary stimuli upon treatment of the animals. When the animals were treated or examined during the night, they were placed in a dark field (<0.05 lux) to avoid light-induced phase change in the circadian rhythm (*25, 26*). Under such circumstances, the rats showed ordinary circadian rhythms of both ACTH and corticosterone concentrations in circulating blood, exhibiting the highest value for both hormones around 8 p.m.-0 a.m. in good agreement with the peak hours in the previous reports (*27, 28*).

Treatments—ACTH-Z (Cortrosyn Z; Organon, Bedford, Holland), a synthetic preparation of $ACTH_{1-24}$ dissolved in 0.9% saline containing 3.1 mg/ml of $ZnCl_2$ and 10 mg/ml of benzyl alcohol was subcutaneously injected into the back of animals at the indicated clock time. The ACTH preparation, which is released slowly from the injected site into circulating blood, has been reported to sustain a high plasma glucocorticoid concentration for nearly 24 h in man (*29*). Metyrapone (Sigma, St. Louis, MO), an inhibitor of glucocorticoid synthesis, was administered to rats by gavage (200 or 400 mg metyrapone/kg body weight) to raise plasma ACTH levels transiently. It has a reported biological half-life in the plasma of rats of about 40-120 min (*30*) and is known to raise the plasma ACTH concentration by a feedback mechanism in the steroidogenic system (*31*), Dexamethasone phosphate, a synthetic glucocorticoid (0.5 mg/kg body weight; Banyu, Tokyo) which effectively suppresses ACTH secretion (*32*), and the vehicle (0.9% saline containing 3.1 mg/ml of $ZnCl_2$, and 10 mg/ml of benzyl alcohol) were also given subcutaneously. The volume of all injections (vehicle, ACTH-Z and dexamethasone) was 400 μl, while that of metyrapone solution administered by gavage was 3 ml.

Detection of Proliferating Cells and Other Immmunohistochemical Stainings—To detect DNA-synthesizing (S-phase) cells in the adrenal cortex, 50 mg/kg body weight of 5-bromo-2′-deoxyuridine (BrdU) (Sigma), dissolved in 500 μl of 0.9% saline containing 0.007 N NaOH, was injected intraperitoneally 1 or 2 h before sacrifice, depending on the experimental protocol. In the experiments to examine circadian rhythms in the cell proliferation, S-phase cells were analyzed at 4-h intervals (±5 min) during a 24-h period. Since the duration of the S-phase in the cell cycle of most tissues, including the adrenal cortex, was known to be 7-8 h (*33, 34*), the interval of 4 h was considered enough to obtain a reasonably good time-resolution. In these experiments, BrdU was injected at 1 h before sacrifice. In the experiments to analyze the effects of ACTH-Z or dexamethasone injection (Figs. 3 and 4, and Table I), BrdU incorporation was examined at longer intervals of up to 8 h, and hence it was injected at 2 h before the sacrifice.

Frozen 6-μm sections of a rat adrenal gland were fixed in a 1:1 mixture of acetone and chloroform, then subjected to the immunohistochemical staining of BrdU-labeled nuclei as described previously (*7*). A mouse monoclonal anti-BrdU antibody was purchased from Becton Dickinson, Mt. View, CA. A biotinylated secondary antibody against mouse IgG and horseradish peroxidase-complexed streptavidin were products of Nichirei, Tokyo, and 3,3′-diaminobenzidine was from Sigma. For the detection of PCNA-expressing cells, 4-μm paraffin sections of the adrenal glands were used as described previously (*8*). A mouse monoclonal anti-PCNA antibody was purchased from DAKO, Copenhagen, Denmark. BrdU- and PCNA-positive cells were counted on the magnified view (×200-400) of transverse sections through the center of the adrenal gland.

Double staining of adrenal sections for pairs of antigens was performed to clarify the functional and spatial relationships among the cells in the adrenal cortex (*35*). To define each adrenocortical zone, adrenal sections were stained simultaneously with antibodies against rat P450aldo, an enzyme specific to zG cells, and $P450_{11\beta}$, an enzyme only present in the zonae fasciculata-reticularis (zFR), as described previously (*7*). In some experiments, antibodies against transcription factor Ad4-binding protein (Ad4BP)/SF-1 (*21, 22*) and P450scc (*23*) were used to detect adrenocortical parenchymal cells in the adrenal sections. Anti-bovine Ad4BP antibody used was a generous gift from Dr. K. Morohashi of the National Institute for Basic Biology (Okazaki).

Other Determinations and Statistics—Plasma ACTH and corticosterone concentrations in rats were measured using commercially available RIA kits (Nihon Mejiphysics, Hyogo, and Amersham, Buckinghamshire, UK, respectively) six times daily at 4-h intervals (±5 min), at 0 a.m., 4 a.m., and 8 a.m., and 0 p.m. (noon), 4 p.m., and 8 p.m. Blood samples were placed in plastic tubes (TERUMO, Tokyo), and centrifuged at 3,000 rpm for 15 min at 4°C immediately after collection.

In counting the number of BrdU-labeled cells in the adrenocortical sections, at least three adrenal sections from an individual adrenal gland were used. The numbers of BrdU-labeled cells obtained from different groups were statistically analyzed using one-way and two-way ANOVA

followed by the *post hoc* Dunn's Procedure for comparison between subjects. Statistical analyses were carried out with the software StatView-J 4.02 for Macintosh. All data are expressed as mean ± SEM. Further details are described under appropriate figure and table legends.

RESULTS

Circadian Rhythms and Regional-Specificity of Cell Proliferation in Rat Adrenal Cortex—Figure 1, A and B, shows the distribution of BrdU-labeled cells in the adrenal cortex of rats killed at 4 p.m. and 4 a.m., respectively. Rats were given an intraperitoneal injection of BrdU at 1 h before sacrifice. BrdU-labeled cells were more abundant at 4 a.m. than at 4 p.m., numbering about 35 per visual field in the former, while less than 15 in the latter. The difference was statistically significant as will be described later. The same situation is depicted more clearly in the magnified views in Fig. 1, C and D. Thus, proliferating cells were more numerous at 4 a.m. than at 4 p.m. under our experimental conditions. The results also showed that most of the BrdU-labeled cells localized in the outer one-third of the adrenal cortex. The outer one-third region contains zG, zU, and the outermost portion of zF, while the inner two-thirds includes the middle and inner portions of zF and the zona reticularis (zR). Distribution of the BrdU-labeled cells did not show a regular pattern in these regions, giving no indication as to the underlying mechanism. However, the labeled cells were all likely to be the adrenocortical parenchymal cells as judged by their positive staining for marker proteins such as transcription factor Ad4BP/SF-1 and P450scc (data not shown) and/or by their shapes and fine structures. Figure 1E shows the localization of P450aldo and $P450_{11\beta}$ in a comparable section, revealing that a cell layer without these enzymes, *i.e.*, zU, is in fact present between the P450aldo-positive zG and the $P450_{11\beta}$-positive zF (7). From comparison of these data, it appeared that a majority of BrdU-labeled cells were in and around zU at 4 p.m., while those newly appeared at 4 a.m. were abundant in the outer portion of zF.

Figure 2, A and B, shows the temporal changes in the number of BrdU- and PCNA-positive cells in comparable sections of the adrenal cortex, respectively. As seen in Fig. 2A, the total number of BrdU-labeled cells countable in the adrenocortical sections showed the highest value at 4 a.m., indicating that DNA-synthesizing cells in the adrenal cortex increased in the early morning ($p<0.01$; data at 4 a.m. *vs.* other times). Cells expressing PCNA, an endogenous marker for the late G_1- and S-phases in the cell cycle, also increased around 4 a.m. ($p<0.01$; data at 4 a.m. *vs.* 0 p.m., 4 p.m., and 8 p.m.) (Fig. 2B). These results are in good agreement with the findings of previous investigators that the proliferation of the cells in rat adrenal cortex has a circadian rhythm (*1-6*). We then compared in detail the distribution of BrdU-labeled cells at 4 a.m. and 4 p.m., and the results obtained were statistically analyzed (Fig. 2C). In this figure, the numbers on the abscissa denote distance expressed as the number of cells from the borderline between zU and the outermost portion of zF: the region between −12 and −5 corresponds to zG, that between −5 and 0 is zU, and the region from 0 through +20 is the outer portion of zF. At 4 p.m., a small number of DNA-synthesizing cells are seen to form twin peaks around zU at the margins with zG and with zF. At 4 a.m., the peak at the margin with zG enlarged slightly but significantly ($p<0.05$; 4 a.m. *vs.* at 4 p.m.), while a burst of cell proliferation occurred at the border with zF, accompanied by a slight shift of the peak toward the inside of the cortex. In the outer portion of zF, the number of BrdU-labeled cells increased about 4-fold between 4 p.m. and 4 a.m. Cells expressing

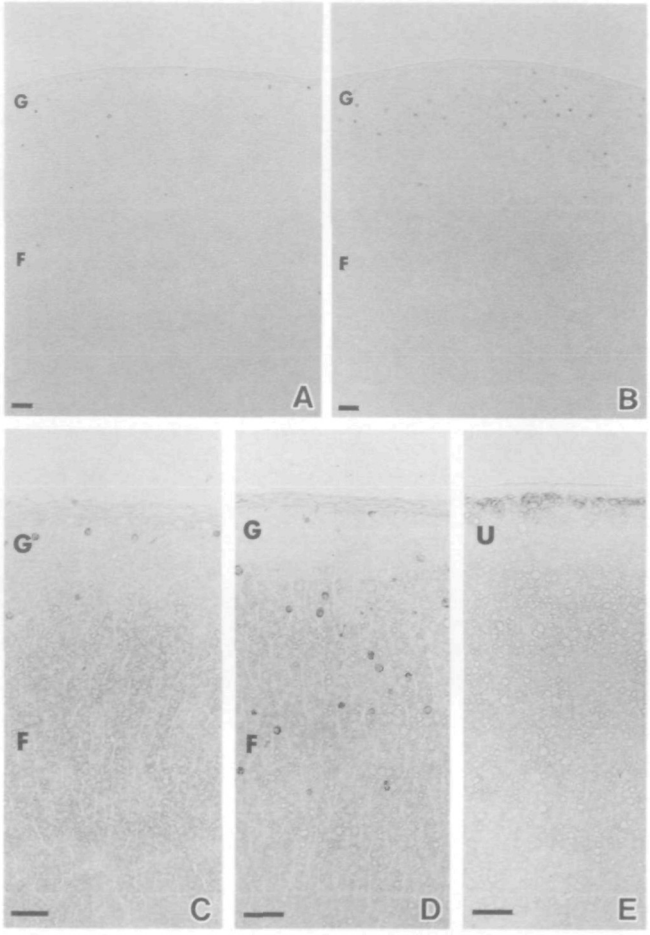

Fig. 1. **Temporal variations in distribution of BrdU-labeled nuclei in rat adrenal cortex.** (A) and (B) show the immunohistochemical distribution of BrdU-labeled nuclei in frozen adrenal sections of the rat killed at 4 p.m. and at 4 a.m., respectively. BrdU was injected into rats 1 h before the sacrifice. Black dots represent BrdU-labeled nuclei. (C) and (D) show magnified views of the immunohistochemical localization of BrdU-labeled nuclei together with that of $P450_{11\beta}$ in the adrenal cortex of rats killed at 4 p.m. and at 4 a.m., respectively. Adrenal sections were stained with anti-BrdU and anti-$P450_{11\beta}$ simultaneously as described in "MATERIALS AND METHODS." Black dots represent BrdU-labeled nuclei, and light black color in the zona fasciculata represents $P450_{11\beta}$-containing cells. (E) shows the immunohistochemical localization of P450aldo and $P450_{11\beta}$ of the adrenal cortex of the rat killed at 4 a.m. Adrenal sections were stained simultaneously with anti-P450aldo and anti-$P450_{11\beta}$ as described before (7). P450aldo-containing cells (dark black color) were observed beneath the capsule within the zona glomerulosa, and $P450_{11\beta}$-containing cells (light black color) were present in the zona fasciculata (for details, see Ref. 7). G and F denote the zona glomerulosa and the zona fasciculata, respectively. U indicates an undifferentiated cell zone which was not stained with either anti-P450aldo or anti-$P450_{11\beta}$ (7). The undifferentiated cell zone is present between the P450aldo-positive zona glomerulosa and the $P450_{11\beta}$- positive zona fasciculata. Bar = 50 μm.

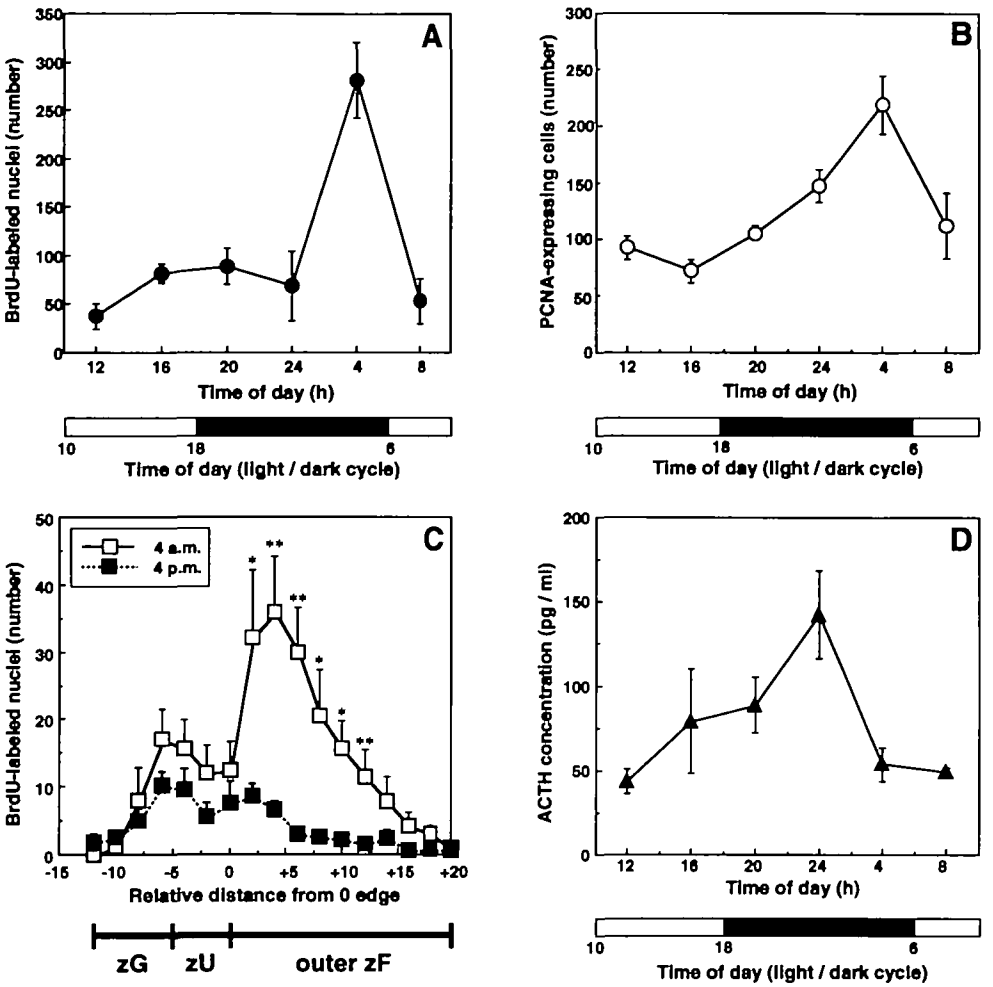

Fig. 2. **Variations in proliferative indices in the adrenal cortex and ACTH concentration in plasma of rats during a 24-h period.** (A) shows the total number of BrdU-labeled nuclei in 6-μm frozen adrenal sections 1 h after injection of BrdU into rats, plotted as a function of the time of day. (B) shows the total number of PCNA-expressing cells in 4-μm paraffin sections of adrenal glands plotted as a function of the time of day. The clock hours of 12, 16, 20, 24, 4, and 8 in the abscissa (X axis) mean 0, 4, 8 p.m., 0, 4, and 8 a.m., respectively. (C) shows the number of BrdU-labeled nuclei in the region from the capsule inward about 30 cells layer in the adrenal cortex of rats killed at 4 a.m. (□) or at 4 p.m. (■). zG, zU, and outer zF show the zona glomerulosa, the undifferentiated cell zone, and the outer portion of the zona fasciculata, respectively. Each point shows the total number of BrdU-positive nuclei within 2 cell layers and is plotted against "Relative distance from 0 edge." "Relative distance from 0 edge" is the number of cell layers from "0" edge in the X axis, and the "0" in X axis is defined as the position of the innermost cell layer of the undifferentiated cell zone in the adrenal cortex. Negative values in the X axis represent the subcapsular portion including the zona glomerulosa and the undifferentiated zone, and positive values represent the outer portion of the zona fasciculata (*$p<0.05$; **$p<0.01$: data at 4 a.m. *vs.* 4 p.m. of the comparable region; all determined by ANOVA followed by the *post hoc* Dunn's Procedure) (D) shows ACTH concentrations in plasma of rats fed on a normal diet plotted as a function of the time of day. Time of day (light/dark cycle) below the X axis shows the light duration (white portion) and dark duration (black portion) in a day as described in "MATERIALS AND METHODS". Values are mean±SEM ($n=9$-10 in A, $n=4$ in B, $n=5$ in C, $n=4$-5 in D).

PCNA also increased significantly in the outer portion of zF at 4 a.m. (data not shown). Thus two distinct sites of cell proliferation exist in rat adrenal cortex, which are distinct from each other in their localization and diural variation.

We then checked the diurnal variation of plasma ACTH concentration, since ACTH affects the size and endocrine functions of the adrenal cortex (*32, 36, 37*). As shown in Fig. 2D, ACTH concentration in circulating blood showed a peak around 0 a.m., declined to a one-third of the peak value at 4 a.m., and began to increase again in the evening. This rhythm roughly coincided with those reported previously (*27, 28*): in comparison with these studies, plasma ACTH concentration began to increase at about the same but reached the maximum concentration approximately 4 h later in our case. This difference might be due to differences in the experimental conditions and/or animal strains used.

Effects of ACTH and Dexamethasone on the Cell Proliferation—The peak of ACTH in circulating blood, observed at midnight, preceded by about 4 h the appearance of a peak of cell proliferation in the adrenal cortex. We accordingly checked effects of plasma ACTH concentration on the adrenocortical cell proliferation by increasing or decreasing it through the administration of exogenous ACTH or dexamethasone to rats.

Figure 3 shows the distribution of BrdU-labeled cells in the outer region of the adrenal cortices from rats injected with a vehicle, a synthetic ACTH (Cortrosyn Z), or dexamethasone. Injections were given at 10 a.m. and the animals were sacrificed at 4 p.m. or 4 a.m. In vehicle-injected rats, few BrdU-labeled cells were detected in the adrenocortical region at 4 p.m. (Fig. 3A) but the number increased by about 3-fold at 4 a.m. (Fig. 3B): the results faithfully reproduced the findings with untreated rats in Fig. 1. In ACTH-injected rats, on the contrary, a significant number of BrdU-labeled cells were observed at 4 p.m. (Fig. 3C), but few were seen at 4 a.m. (Fig. 3D). The decrease in BrdU-labeled cells was particularly remarkable in the outer zF. It should be noted that the Cortrosyn Z used here and in the following experiments is a long-acting preparation, which is released slowly into the blood and maintains a high plasma ACTH level high for about 24 h. We confirmed that the concentration of corticosterone, a measure of

the ACTH concentration in the blood (*29*), was as high as about 100 μg/dl between 2 p.m. and 4 a.m. This value was 2-4 times higher than those of control rats during the same period. The very small number of BrdU-positive cells in Fig. 3D indicated that the adrenocortical cells were not proliferative, despite the high concentration of ACTH in the systemic circulation.

In dexamethasone-injected rats (Fig. 3, E and F), very few BrdU-labeled cells were found both at 4 p.m. (6 h after the injection) and 4 a.m. (18 h after the injection). The paucity of proliferating cells upon administration of dexamethasone was confirmed by detecting the expression of PCNA, another marker of cell proliferation (data not shown). Since the administration of dexamethasone causes the depletion of plasma ACTH concentration, these data suggest that ACTH in the circulating blood is necessary for the cell proliferation in the adrenal cortex. The significance of these results will be discussed later.

The results of similar experiments are evaluated as a function of time in Fig. 4. ACTH-Z was also administered at 10 a.m., and rats were sacrificed at 4 p.m., 8 p.m., and 4 a.m. BrdU was injected 2 h before sacrifice. As seen in Fig. 4A, the number of proliferating cells increased by over 4-fold at 5 h after the ACTH-injection, then declined to the basal level within the next 4 h, and thereafter maintained the same level until the end of the experiment (at 17 h after

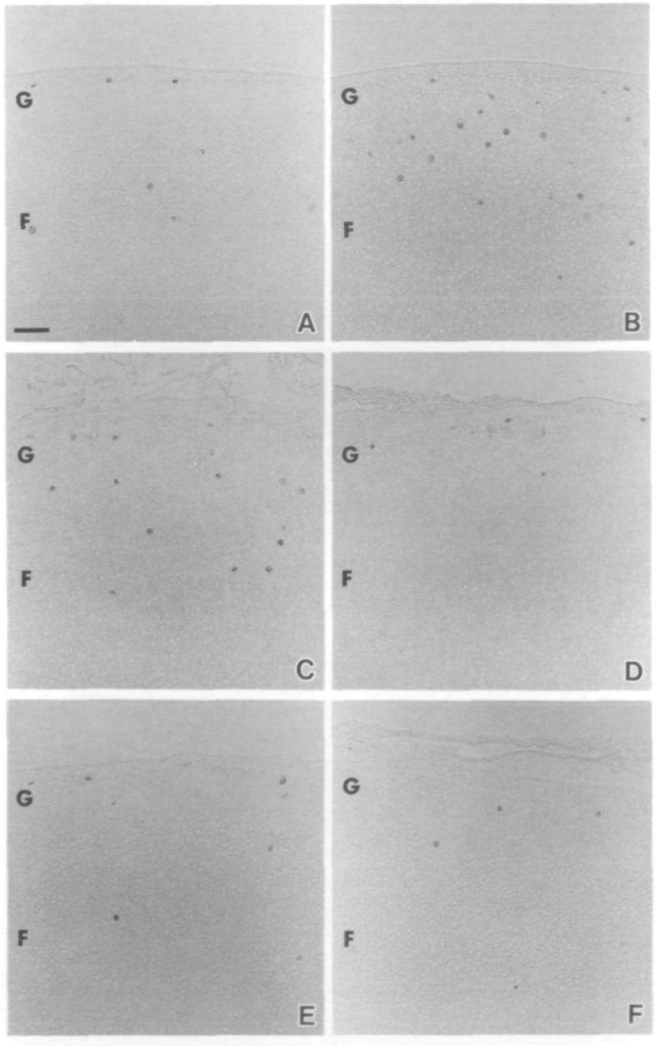

Fig. 3. **Immunohistochemical distribution of BrdU-labeled nuclei in the adrenal cortices after vehicle-, long acting ACTH (ACTH-Z)-, and dexamethasone-injection.** (A), (C), and (E), respectively, show the immunohistochemical distribution of BrdU-labeled nuclei in the adrenal cortex from rats 6 h (at 4 p.m.) after the injection of vehicle, ACTH (5 IU/kg body weight), and dexamethasone at 10 a.m. (B), (D), and (F), respectively, show the distribution of BrdU-labeled nuclei in the adrenal cortex from rats 18 h (at 4 a.m.) after the injection of vehicle, ACTH (5 IU/kg body weight), and dexamethasone. BrdU was injected into rats 2 h before the sacrifice. Black dots indicate BrdU-positive nuclei. G and F denote zona glomerulosa and the zona fasciculata, respectively. Bar = 50 μm.

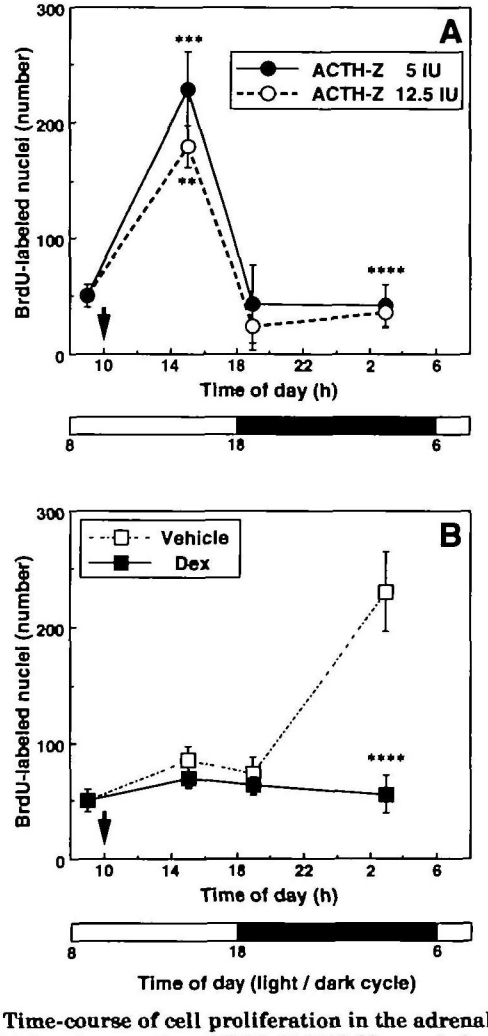

Fig. 4. **Time-course of cell proliferation in the adrenal cortex after vehicle-, long-acting ACTH (ACTH-Z)-, and dexamethasone-injection.** The numbers of BrdU-labeled nuclei in 6-μm frozen adrenal sections were plotted as a function of clock time after injection of ACTH-Z (● and ○ in A, respectively, for injection of 5 IU ACTH and 12.5 IU ACTH/kg body weight), dexamethasone (Dex) (■ in B), and the vehicle (□ in B) to rats at 10 a.m. (indicated by arrows). BrdU was injected into rats 2 h before the sacrifice, and the numbers of BrdU-labeled nuclei counted in the adrenal sections were plotted as those at 1 h after injection of BrdU in the figure. Values are mean ± SEM (n = 3-6). [$^{**}p < 0.01$ or $^{***}p < 0.001$: data on ACTH (12.5 IU) or ACTH (5 IU)-injected *vs.* vehicle-injected rats at each time point; $^{****}p < 0.0001$: data on ACTH or dexamethasone-injected *vs.* vehicle-injected rats at each time point; all determined by ANOVA followed by the *post hoc* Dunn's Procedure].

TABLE I. **BrdU-labeled nuclei in the adrenal cortices of rats treated with ACTH and dexamethasone.** The number of BrdU-labeled nuclei in each adrenocortical zone 6 h (4 p.m.) and 18 h (4 a.m.) after the injection was counted in vehicle-, synthetic ACTH- and dexamethasone-injected rats. The data in each column show the average number of BrdU-labeled nuclei within the indicated region in an adrenal section. The rats were sacrificed at the indicated time. BrdU was administered intraperitoneally 2 h before sacrifice. Differences between the groups were statistically analyzed using ANOVA followed by the *post hoc* Dunn's Procedure.

Group	Injection at 10 a.m.	Outer region (zona glomerulosa & undifferentiated cell zone)		Inner region (zona fasciculata-reticularis)	
		6 h after injection (4 p.m.)	18 h after injection (4 a.m.)	6 h after injection (4 p.m.)	18 h after injection (4 a.m.)
		Number of nuclei (mean±SEM)		*Number of nuclei (mean±SEM)*	
1	Vehicle	52.0±7.5	55.0±10.2	33.7±7.3	175.7±26.2[a]
2	ACTH (5 IU)	51.0±7.9	30.8±11.6	163.2±33.2[e]	8.0±2.0[a,f]
3	ACTH (12.5 IU)	55.6±18.4	32.8±9.1	126.7±12.2[d]	6.3±2.7[a,f]
4	Dexamethasone	34.4±4.3	9.3±3.1[b,c]	40.0±7.5[g,h]	35.7±13.0[f]

[a] $p<0.01$ *vs.* data 4-6 h after the same treatment. [b] $p<0.001$ *vs.* data 4-6 h after the same treatment. [c] $p<0.05$ *vs.* data at the same time of the vehicle-treated rats. [d] $p<0.01$ *vs.* data at the same time of the vehicle-treated rats. [e] $p<0.001$ *vs.* data at the same time of the vehicle-treated rats. [f] $p<0.0001$ *vs.* data at the same time of the vehicle-treated rats. [g] $p<0.01$ *vs.* data at the same time of the ACTH (5 IU)-treated rats. [h] $p<0.01$ *vs.* data at the same time of the ACTH (12.5 IU)-treated rats.

the injection). It should be noted that the time required for the onset of cell proliferation after the ACTH stimulus was almost the same to that observed in the diurnal rhythm described in Fig. 2: The ACTH surge preceded the cell proliferation by 4-6 h.

In contrast, injection of dexamethasone at 10 a.m. (0.5 mg/kg body weight) caused neither increase nor decrease in BrdU incorporation for at least 17 h after the administration, but kept a basal level of cell proliferation throughout the period (Fig. 4B, closed squares). The peak of cell proliferation observed in the early morning in vehicle-treated rats (Fig. 4B, open squares) and under our standard conditions (Fig. 2A) was abolished. After the injection of dexamethasone, the plasma ACTH concentration diminished to less than 5 pg/ml for at least 18 h, which was about one-tenth of the value 50 pg/ml under our standard experimental conditions. It should be noted, however, that the dexamethasone-treatment did not cause a decrease in the basal level of cell proliferation in the whole adrenal cortex.

Zone-Specific Proliferation and the Sensitivities toward ACTH and Dexamethasone—Effects of ACTH and dexamethasone on the adrenocortical cell proliferation were further analyzed and the results are summarized in Table I. Here we divided the adrenal cortex into two regions, the outer region, which includes zG and zU, and the inner region, which contains the whole zFR. In the outer region at 6 h after the injection, there was no statistically significant difference in the BrdU-labeling in the adrenal cortices from rats treated with either vehicle, two doses of ACTH, or dexamethasone. The lower value in the dexamethasone-treated animals was significantly different. At 18 h after the injection (4 a.m.), there was still no significant difference in the BrdU-labeling between vehicle- and ACTH-treated animals, whereas a large difference was observed in the dexamethasone-treated rats: the proliferative index in the outer region, *i.e.*, in zG and zU, decreased to less than 1/6 of that in the vehicle-treated animals (9.3±3.1 *vs.* 55.0±10.2). In other words, it fell below the basal level. The results were not only in accordance with those in Figs. 3 and 4, but suggested that ACTH was required for the maintenance of the basal level of cell proliferation in the outer region of the adrenal cortex.

On the other hand, the proliferative index in the inner region of the adrenal cortex was increased significantly at 6 h after the injection of ACTH (4 p.m.) ($p<0.001$ or $p<$

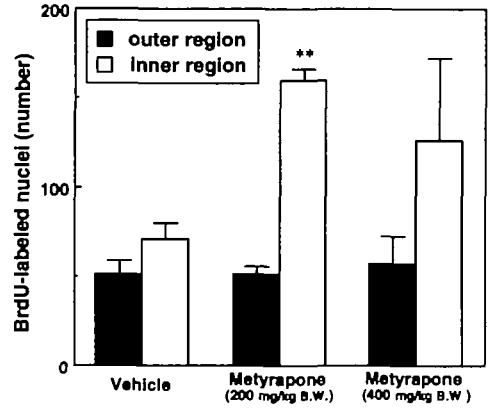

Fig. 5. **Number of BrdU-labeled nuclei in outer- or inner-region of the adrenal cortex in rats after the administration of metyrapone by gavage.** Closed bars and open bars show the numbers of BrdU-labeled nuclei in outer and inner regions of the frozen adrenal sections 6 h (4 p.m.) after the administration of metyrapone to rats by gavage at 10 a.m., respectively. The outer region shows the region containing the zona glomerulosa and the undifferentiated zone. The inner region shows the zonae fasciculata-reticularis. BrdU was injected into rats at 3 p.m., 1 h before the sacrifice. Vehicle, Metyrapone 200 mg/kg B.W. and Metyrapone 400 mg/kg B.W. on the X axis show groups administered H_2O, 200, and 400 mg metyrapone/kg body weight, respectively. Values are mean± SEM ($n=4$-5). (**$p<0.01$: data on Metyrapone (200 mg/kg body weight) *vs.* Vehicle; all determined by ANOVA followed by the *post hoc* Dunn's Procedure).

0.01; *vs.* vehicle-injected rats), in good agreement with the results shown in Figs. 3 and 4. However, a higher dose of ACTH (12.5 IU) showed a smaller effect than the lower dose (5 IU). In dexamethasone-injected rats, the data at 6 h after the injection were unchanged from those of the animals treated with the vehicle, although plasma ACTH level was expected to be less than 1/10 of that of the ordinary animals (see previous section). At 18 h after the injection, the vehicle-treated animals showed the highest proliferative index owing to the daily circadian rhythm, while the ACTH-injected animals had lost almost all the proliferative activities, being below the basal level (8.0± 2.0 and 6.3±2.7 *vs.* 33.7±7.3) (Table I). On the other hand, dexamethasone-treated animals maintained well the basal proliferative activity (35.7±13.0), which was in a

marked contrast to the decrease found in the outer region (9.3 ± 3.1). These results revealed that differences exist in the mechanism of cell proliferation between the outer region composed of zG plus zU and the inner region composed of zF and zR.

Effects of Metyrapone on the Cell Proliferation—In the next series of experiments, we gave rats metyrapone, which inhibits the activity of deoxycorticosterone 11β-hydroxylase, thereby promoting the secretion of ACTH from hypophyses and resulting in a high plasma ACTH concentration (*31*).

When plasma ACTH level in rats was raised by giving a high dose of metyrapone by gavage at 10 a.m., a significant increase in the number of replicating cells were observed in the outer portion of zF at 2-4 p.m., *i.e.*, 4-6 h after the administration. BrdU-labeled cells numbered 30-50 in specimens of comparable size to those in Fig. 1A, where the number of BrdU-labeled cells was below 15. Figure 5 compares the number of BrdU-labeled cells in the outer and inner regions of the adrenal cortices in rats administered metyrapone by gavage 6 h before sacrifice. Significant increases in the number of BrdU-labeled cells were seen in the inner region (open squares), but not in the outer region (closed squares), in good agreement with the results obtained by the administration of synthetic ACTH in the previous section (Table I).

DISCUSSION

Many eukaryotic cells show a circadian rhythm in their cell proliferation, with a periodicity of roughly 24 h both *in vivo* and *in vitro* (*6, 38, 39*). Here we have shown that rat adrenocortical cells also replicate with a circadian periodicity, as judged by the number of DNA-synthesizing (S-phase) cells measured by the incorporation of BrdU into the cell-nuclei and/or the expression of proliferating cell nuclear antigen (PCNA). The S-phase cells thus observed reached the maximum number at 4 a.m. These results confirm the previous findings of a circadian rhythm in the adrenocortical cytogenesis of rodents, with a proliferative peak at midnight (*2, 4*), or in the morning (*3, 6*). It has also been reported that most proliferating cells were found in an outer region of the adrenal cortex, from the subcapsular to the outer portion of zF (*40-42*). In these studies, however, it was difficult to identify accurately the site(s) of cell proliferation and the pedigree of the proliferating cells, since no cell marker specific to each type of cells in the tissue was then available. Furthermore, the presence of zU in its presently identified form was not known. In this study, we identified the sites of cell proliferation as two distinct regions around zU (Fig. 2C): (i) the region containing the outer portion of zF and the inner edge of zU, and (ii) the region around the boundary between zG and zU. Cell proliferation in the former region varied greatly depending on the clock time, showing the maximum number of the S-phase cells at 4 a.m., while that in the latter region showed little or no circadian variation. Thus at least two distinct sites for cell proliferation around zU were recognized in the adrenal cortex of adult rats.

A number of factors have been shown to affect the secretion of adrenocortical hormones and the proliferation of cells in the adrenal cortex: these include cytokines such as FGF and IGF (*31, 43-45*), and hormones such as ACTH, angiotensin II, sex hormones, and melatonin (*7, 8, 19, 32, 46*). In this study, we examined effects of ACTH. The circadian variation in cytogenesis in the inner region, *i.e.*, the outer portion of zF plus inner edge of zU, seemed to be modulated by the plasma concentration of ACTH. In this connection, it should be noted that injection of short-acting ACTH twice in 24 h disturbed the circadian rhythm in mice, as assessed by the nuclear volume of zF cells (*2*).

We found that a prominent rise in the plasma ACTH concentration preceded the cell proliferation maximum by 4-6 h. This temporal relationship was confirmed by the administration of exogenous ACTH or by the metyrapone-treatment to increase the plasma ACTH concentration. These treatments brought about enhanced cytogenesis in the inner region at 4-6 h after the rise in plasma ACTH concentration. It was not clear, however, whether the rise in plasma ACTH concentration itself or the modulation of the circadian rhythm in the plasma ACTH concentration triggered the cell proliferation. On the other hand, increased ACTH concentration had little effect on the cell replication in the outer region, suggesting a difference in sensitivities and/or responses to ACTH between the outer and the inner sites of cytogenesis in the adrenal cortex.

When dexamethasone was administered, the cell replication in the outer region diminished to less than 1/6 of the control level at 18 h after the injection, suggesting that depletion of ACTH by the dexamethasone treatment severely depressed the basal level of cell proliferation in this area. ACTH is probably necessary to sustain the minimum level of proliferation required for tissue maintenance, even though its increase over the basal level does not enhance appreciably the cytogenesis in this region. This finding is in contrast with the effect of dexamethasone-treatment on the inner region, where the basal level of cell replication was maintained even in the ACTH-depleted state. A high concentration of ACTH for a prolonged period during the day depressed the cell proliferation in this region below the baseline level (see Table I).

The two distinct cell proliferation sites described here are of special importance in considering the mechanism(s) for the formation and maintenance of the zonal structure and zone-specific functions of the adrenal cortex. Previous works have shown that the replicating cells, such as those labeled with ^{3}H-thymidine, were initially found in the outer portion of the adrenal gland, moved slowly toward the inner portion, and reached the inner end of zF after about 1 mo (*42, 47*). It has been thus postulated that the adrenocortical cytogenesis occurs in the outer region of the cortex, the cells born there migrate centripetally, and degenerate at the boundary of the zR to the medulla; and this postulate has been called "cell migration theory" or "escalatory theory" (*40, 48*). These studies implicitly assume a single site (or a region) of cell production, where the cells are born as zG cells, transformed into zF cells, and finally become zR cells as they move up the escalator. However, our results show the presence of two distinct proliferation sites in the adrenal cortex, which are located in close proximity to each other but differ in their responses toward external stimuli. It is possible to speculate from these results and our previous findings (*7, 8*) that the outer site (a region from the inner half of zG to near the outer margin of zF) provides the cells to zG, and the inner site (the outer one-fourth portion of zF) produces the cells for both zF and zR. This

postulate is consistent with the "proliferative intermediate zone hypothesis" (*49*): that cells in the intermediary zone between zG and zF provide daughter cells to both zG and zF. As mentioned, this intermediary zone can be considered to correspond to zU described herein, and we have shown that the two sites for the cell proliferation localize at either side of zU. In this connection, it should be noted that the cells around the stem cell layer are in general more active with respect to cell proliferation than those in the stem cell layer (*50*), and that the number of S-phase cells in zU is smaller than in the two proliferation sites nearby. Therefore, we tentatively support the "proliferative intermediate zone hypothesis" rather than the "cell migration theory" or "escalatory theory" of cell renewal and maintenance in the adrenal cortex. We do, however, need to retain the latter hypothesis. Without the migration of the cells from zF and their conversion to zR cells on the escalator, the supply of cells to zR would be difficult. Without this, we have to consider revival of the "zonal theory" (*51*), that the cells in each zone are born and die in the same zone. Experiments to clarify these points as well as to explore the regulatory mechanism(s) of cell proliferation in the adrenal cortex are underway.

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REFERENCES

1. Mühlemann, H.R., Marthaler, T.M., and Loustalot, P. (1955) Daily variations in mitotic activity of adrenal cortex, thyroid and oral epithelium of the rat. *Proc. Soc. Exp. Biol.* **90**, 467-468
2. Kirillov, O.I. and Kurilenko, L.A. (1979) Effect of ACTH on circadian periodicity of nuclear volume and mitotic division in the zona fasciculata externa of the adrenal cortex of mice. *Int. J. Chronobiol.* **6**, 51-55
3. Ueberberg, H., Stolz, W., and Trieb, G. (1982) Autoradiographic studies of the diurnal rhythm of the cell proliferation in the adrenal cortex of the rat. *Path. Res. Pract.* **173**, 183-190
4. Kirillov, O.I. and Popova, S.B. (1985) Adrenal cortex of rat: diurnal variation of nuclear volumes and mitotic index of cells in zona fasciculata. *Exp. Clin. Endocrinol.* **86**, 375-377
5. McNicol, A.M., Penman, I.D., and Duffy, A.E. (1989) Age-related variation in circadian rhythms of mitosis in the adrenal cortex of the male rat. *J. Endocrinol.* **120**, 307-310
6. Messier, B. and Leblond, C.P. (1960) Cell proliferation and migration as revealed by radioautography after injection of ^{3}H-thymidine into male rats and mice. *Am. J. Anat.* **106**, 247-285
7. Mitani, F., Suzuki, H., Hata, J., Ogishima, T., Shimada, H., and Ishimura, Y. (1994) A novel cell layer without corticosteroid-synthesizing enzymes in rat adrenal cortex: histochemical detection and possible physiological role. *Endocrinology* **135**, 431-438
8. Mitani, F., Ogishima, T., Miyamoto, H., and Ishimura, Y. (1995) Localization of P450aldo and P450$_{11\beta}$ in normal and regenerating rat adrenal cortex. *Endocrine Res.* **21**, 413-423
9. Ogishima, T., Mitani, F., and Ishimura, Y. (1989) Isolation of aldosterone synthase cytochrome P450 from zona glomerulosa mitochondria of rat adrenal cortex. *J. Biol. Chem.* **264**, 10935-10938
10. Ogishima, T., Shibata, H., Shimada, H., Mitani, F., Suzuki, H., Saruta, T., and Ishimura, Y. (1991) Aldosterone synthase cytochrome P450 expressed in the adrenals of patients with primary aldosteronism. *J. Biol. Chem.* **266**, 10731-10734
11. Mitani, F. and Horie, S. (1970) Studies on P-450. VII. Reconstitution of a steroid 11β-hydroxylation system using a P450 preparation extracted and partially purified from adrenocortical mitochondria. *J. Biochem.* **68**, 529-542
12. Mitani, F., Ichiyama, A., Masuda, A., and Ogata, I. (1975) Enzymic studies on adrenocortical deoxycorticosterone 11β-hydroxylase system. *J. Biol. Chem.* **250**, 8010-8015
13. Reiss, M., Balint, J., Oestreicher, F., and Aronson, V. (1936-7) Zur morphogenetischen Wirkung und biologishen Eichung der kortikotropen Wirkstoffes. *Endocrinolgie* **18**, 1-10
14. Hall, K. and Korenchevsky, V. (1937) Histological changes produced by castration and by sex hormones in the adrenals of normal and of castrated male rats. *Nature* **140**, 318
15. Deane, H.W. and Greep, R.O. (1946) A morphological and histochemical study of the rat's adrenal cortex after hypophysectomy, with comments on the liver. *Am. J. Anat.* **79**, 117-145
16. Hartroft, P.M. and Eisenstein, A.B. (1957) Alterations in the adrenal cortex of the rat induced by sodium deficiency: correlation of histologic changes with steroid hormone secretion. *Endocrinology* **60**, 641-651
17. Nicander, L. (1952) Histological and histochemical studies on the adrenal cortex of domestic laboratory animals. *Acta. Anat.* [Suppl] **14**, 16
18. Cater, D.B. and Lever, J.D. (1954) The zona intermedia of the adrenal cortex. A correlation of possible functional significance with development, morphology and histochemistry. *J. Anat.* **88**, 437-454
19. Cain, A.J. and Harrison, R.G. (1950) Cytological and histochemical variation in the adrenal cortex of the albino rat. *J. Anat.* **84**, 196-226
20. Mitchell, R.M. (1948) Histological changes and mitotic activity in the rat adrenal during postnatal development. *Anat. Rec.* **101**, 161-185
21. Morohashi, K., Iida, H., Nomura, M., Hatano, O., Honda, S., Tsukiyama, T., Niwa, O., Hara, T., Takakusu, A., Shibata, Y., and Omura, T. (1994) Functional difference between Ad4BP and ELP, and their distribution in steroidogenic tissues. *Mol. Endocrinol.* **8**, 643-653
22. Luo, X., Ikeda, Y., and Parker, K.L. (1994) A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. *Cell* **77**, 481-490
23. Mitani, F., Shimizu, T., Ueno, R., Ishimura, Y., Izumi, S., Komatsu, N., and Watanabe, K. (1982) Cytochrome P450$_{11\beta}$ and P450scc in adrenal cortex: zonal distribution and intramitochondrial localization by the horseradish peroxidase-labeled antibody method. *J. Histochem. Cytochem.* **30**, 1066-1074
24. Mitani, F., Mukai, K., Miyamoto, H., Suematsu, M., and Ishimura, Y. (1999) Development of functional zonation in rat adrenal cortex. *Endocrinology* **140**, 3342-3353
25. De Coursey, P.J. (1960) Daily light sensitivity rhythm in a rodent. *Science* **131**, 33-35
26. Ding, J.M., Buchanan, G.F., Tischkau, S.A., Chen, D., Kuriashkina, L., Faiman, L.E., Alster, J.M., McPherson, P.S., Campbell, K.P., and Gillette, M.U. (1998) A neuronal ryanodine receptor mediates light-induced phase delays of the circadian clock. *Nature* **394**, 381-384
27. Kaneko, M., Hiroshige, T., Shinsako, J., and Dallman, M.F. (1980) Diurnal changes in amplification of hormone rhythms in the adrenocortical system. *Am. J. Physiol.* **239**, R309-R316
28. Fischman, A.J., Kastin, A.J., Graf, M.V., and Moldow, R.L. (1988) Constant light and dark affect the circadian rhythm of the hypothalamic-pituitary-adrenal axis. *Neuroendocrinology* **47**, 309-316
29. Tsuji, S., Kouzuki, Y., Taniguchi, H., Takami, Y., Roujin, M., and Yasui, H. (1969) Clinical studies on synthetic ACTH (β^{1-24} peptide) (in Japanese). *Shinryo* **22**, 612-620
30. Suzuki, Y. (1972) Sex difference in hypertensive action of metopirone in relation to its plasma levels in the rat. *Endocrinology* **90**, 924-930
31. Coulter, C.L., Goldsmith, P.C., Mesiano, S., Voytek, C.C., Martin, M.C., Han, V.K.M., and Jaffe, R.B. (1996) Functional maturation of the primate fetal adrenal in vivo. *Endocrinology* **137**, 4487-4498
32. Mitani, F., Miyamoto, H., Mukai, K., and Ishimura, Y. (1996)

Effects of long term stimulation of ACTH- and angiotensin II-secretions on the rat adrenal cortex. *Endocrine Res.* **22**, 421-431
33. Nowakowski, R.S., Lewin, S.B., and Miller, M.W. (1989) Bromodeoxyuridine immunohistochemical determination of the lengths of the cell cycle and the DNA-synthetic phase for an anatomically defined population. *J. Neurocytol.* **18**, 311-318
34. Malendowicz, L.K. and Jachimowicz, B. (1982) Sex differences in adrenocortical structure and function. XI. *Cell Tissue Res.* **227**, 651-657
35. Nakane, P. (1968) Simultaneous localization of multiple tissue antigens using the peroxidase-labeled antibody method: a study on pituitary glands of the rat. *J. Histochem. Cytochem.* **16**, 557-560
36. Saez, J.M., Morera, A.M., and Gallet, D. (1977) Opposite effects of ACTH and glucocorticoids on adrenal DNA synthesis *in vivo*. *Endocrinology* **100**, 1268-1275
37. Pudney, J., Price, G.M., Whitehouse, B.J., and Vinson, G.P. (1984) Effects of chronic ACTH stimulation on the morphology of the rat adrenal cortex. *Anat. Rec.* **210**, 603-615
38. Sassone-Corsi, P. (1998) Molecular clocks: mastering time by gene regulation. *Nature* **392**, 871-874
39. Smaaland, R. (1996) Circadian rhythm of cell division. *Prog. Cell Cycle Res.* **2**, 241-266
40. Gottschau, M. (1883) Struktur und Embryonale Entwickelung der Nebennieren bei Saugetieren. Archiv für Anatomie und Entwickelungsgeschichte. *Anatomischer Abteilung* **9**, 412-458
41. Estivariz, F.E., Lowry, P.J., and Jackson, S. (1992) Control of adrenal growth in *The Adrenal Gland, Second Edition, Comprehensive Endocrinology revised series* (James, V.H.T., ed.) pp. 43-70, Raven Press, New York
42. Belloni, A.S., Mazzocchi, G., Meneghelli, V., and Nussdorfer, G.G. (1978) Cytogenesis in the rat adrenal cortex: evidence for an ACTH-induced centripetal cell migration from zona glomerulosa. *Arch. Anat. Hist. Embr. norm. et. exp.* **61**, 195-206
43. Lotfi, C.F.P., Todorovic, Z., Armelin, H.A., and Schimmer, B.P. (1997) Unmasking a growth-promoting effect of the adrenocorticotropic hormone in Y-1 mouse adrenocortical tumor cells. *J. Biol. Chem.* **272**, 29886-29891
44. Hornsby, P.J. (1985) Regulation of adrenocortical cell proliferation in culture. *Endocrine Res.* **10**, 259-281
45. Liakos, P., Bourmeyster, N., Defaye, G., Chambaz, E.M., and Bottari, S.P. (1997) ANG II AT1 and AT2 receptors both inhibit bFGF-induced proliferation of bovine adrenocortical cells. *Am. J. Physiol.* **273**, C1324-1334
46. Sewerynek, E. and Lewinski, A. (1989) Melatonin inhibits mitotic activity of adrenocortical cells in vivo and in organ culture. *J. Pineal. Res.* **7**, 1-12
47. Zajicek, G., Ariel, I., and Arber, N. (1986) The streaming adrenal cortex: direct evidence of centripetal migration of adrenocytes by estimation of cell turnover rate. *J. Endocrinol.* **111**, 477-482
48. Nussdorfer, G.G. (1986) Cytogenesis in the adrenal cortex in *Cytophysiology of the Adrenal Cortex. International Review of Cytology* (Bourine, G.H. and Danielli, J.F., eds.) Vol. 98, pp. 319-330, Academic Press, New York
49. Idelman, S. (1978) The structure of the mammalian adrenal cortex in *General, Comparative and Clinical Endocrinology of the Adrenal Cortex* (Chester Jones, I. and Henderson, I.W., eds.) Vol. 2, pp. 1-199, Academic Press, New York
50. Morrison, S.J., Shah, N.M., and Anderson, D.J. (1997) Regulatory mechanisms in stem cell biology. *Cell* **88**, 287-298
51. Swann, H.G. (1940) Pituitary-adrenocortical relationship. *Physiol. Rev.* **20**, 493-521