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Adrenocortical and Gonadal Expression of the Mammalian *Ftz-F1* Gene Encoding Ad4BP/SF-1 Is Independent of Pituitary Control[1]

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Ad4BP/SF-1 is a transcription factor essential for the development of the adrenal gland and the gonads as well as for the maintenance of their functions through regulating tissue-specific gene transcription. In the whole body, hypothalamo-pituitary-gonadal and -adrenal axes are known to play prominent roles in mediating the function of the gonads and adrenal. In this study, the effects of the tropic peptide hormones secreted by the pituitary on the regulation of the rat *Ftz-F1* (*rFtz-F1*) gene encoding Ad4BP/SF-1 were investigated. Immunochemical studies revealed that Ad4BP/SF-1 was expressed even in the adrenal cortex of hypophysectomized rats. Such persistent expression of Ad4BP/SF-1 was also observed in the testes and ovaries of the hypophysectomized animals. In contrast to Ad4BP/SF-1, the expressions of steroidogenic P450s were reduced significantly. The transcriptional activities of the endogenous and transfected *rFtz-F1* genes were examined with Y-1 and I-10 cells derived from mouse adrenocortical and testicular Leydig cell tumors, respectively. Neither gene appeared to be activated significantly by cAMP, whereas both endogenous and exogenous *CYP11A* genes encoding P450(SCC) were activated. Taken together, these observations indicate that the expression of the *rFtz-F1* gene is mainly regulated by a mechanism independent of the neuroendocrine axes.

Key words: adrenal cortex, Ad4BP/SF-1, *Ftz-F1*, gonad, nuclear receptor.

Steroid hormones are synthesized in the testis, ovary, and adrenal cortex by the function of six forms of steroidogenic cytochrome P450. A nuclear receptor, Ad4BP/SF-1, was originally identified as the transcription factor regulating the steroidogenic *CYP* (P450) gene in a tissue-specific manner (*1-7*). Indeed, the distribution of Ad4BP/SF-1 was found to overlap with those of the steroidogenic P450s (*8, 9*). However, recent histochemical and *in situ* hybridization studies have revealed the presence of Ad4BP/SF-1 in the pituitary gonadotroph and ventromedial hypothalamic nucleus (VMH) in addition to the steroidogenic tissues (*10, 11*). Although the genes governed by Ad4BP/SF-1 in the VMH remain to be elucidated, recent studies have shown that the *LH* (luteinizing hormone) and *FSH* (follicle stimulation hormone) genes are regulated by this nuclear receptor in the pituitary gonadotroph (*12-15*).

The hypothalamo-pituitary-gonadal and -adrenal axes play pivotal roles in maintenance of the reproductive function and homeostasis of glucose and electrolyte concentrations in internal fluids. For the functions of these axes, hypophysiotropic substances, gonadotropin releasing hormone (GnRH), and adrenocorticotropin releasing hormone (CRH), are discharged from particular neurons in the hypothalamic central nervous system. These neuropeptide hormones are transported by the portal vascular system and stimulate the functions of gonadotrophs and corticotrophs of the anterior pituitary. Subsequently, gonadotropins (FSH and LH) and adrenocortical tropic hormone (ACTH) are secreted by the corresponding pituitary trophs (*16*). In response to stimulation by the pituitary tropic hormones, peripheral tissues such as the gonads and adrenal cortex synthesize and secrete sex steroids and corticosteroids, respectively (*17-19*). Contrary to this signal flow from the central to the peripheral tissues, steroid hormones exert inhibitory feedback effects on the secretion of the tropic peptide hormones by suppressing the release of the hypophisiotropic substances (*16*). Consequently, all the tissues constituting each axis display their functions in a coordinated manner by secreting a variety of circulating signaling factors.

Disruption studies of the *rFtz-F1* gene encoding Ad4BP/SF-1 have demonstrated the crucial role of this factor in adrenal and gonadal differentiation by showing that the gene-disrupted mice develop neither gonads nor adrenal

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glands (*11, 20, 21*). Simultaneous disappearance of these distinct tissues from the mice suggests a novel ontogenical relationship between the tissue components of the hypothalamo-pituitary-gonadal and -adrenal axes during their differentiation. This assumption was clearly visualized by a recent immunohistochemical study using an antiserum to Ad4BP/SF-1, in which the gonads and adrenal cortex were shown to originate from an identical cell-population designated the "adreno-genital primordium" (*22, 23*). Because of the functional significance of Ad4BP/SF-1, elucidation of the transcriptional regulation of the *rFtz-F1* gene is essential to understanding the differentiation process of the steroidogenic tissues. In the present study, the expression of the *rFtz-F1* gene was investigated with hypophysectomized animals and activated cultured cells by cAMP to assess the effects of the pituitary tropic hormones on the gene transcription. The expression of Ad4BP/SF-1 in the hypophysectomized animals and cultured cells indicated that the *rFtz-F1* gene is independent of control by the tropic hormones from the pituitary.

MATERIALS AND METHODS

Immunohistochemical and Immunochemical Analyses—Hypophysectomized adult Wistar rats (8 weeks) of both sexes were purchased from the Imamichi Institute for Animal Reproduction (Niihari). Four weeks after the hypophysectomy, the adrenal glands, testes, and ovaries were excised from the hypophysectomized and sham-operated animals and fixed with 4% paraformaldehyde-0.1 M potassium phosphate (pH 7.0) at 4°C overnight. After dehydration, the fixed tissues were embedded in paraffin and sectioned. Sections of 5 μm were mounted on slides, deparaffinized, rehydrated, then boiled in 10 mM citrate (pH 6.0) for 10 min using a microwave to retrieve antigens for detection of Ad4BP/SF-1. The boiling was omitted for detection of P450(SCC). To avoid nonspecific reaction of endogenous peroxidases, the sections were incubated with 0.3% H_2O_2 in methanol for 30 min at room temperature, then incubated with 2% skim milk in 10 mM Tris-HCl (pH 7.5) containing 0.8% NaCl for 1 h. After overnight incubation with 1:1,500 diluted rabbit antiserum to bovine Ad4BP/SF-1 (*4*) or 1:1,000 diluted rabbit antiserum to human P450(SCC) in a humidified chamber at 4°C, the sections were washed three times with 10 mM Tris-HCl (pH 7.5) containing 0.8% NaCl. The antibody-antigen complexes were detected by the streptavidin-biotin-peroxidase method using a Histofine kit (Nichirei, Tokyo). Colorimetric reaction was performed as described (*24*).

For immunoblot analyses, the adrenal cortex was isolated from the adrenal glands under the microscope, while whole tissues were used in the case of the testis and ovary. These tissues were lysed with 50 mM Tris-HCl (pH 7.5) containing 2% SDS and subjected to sonication to disrupt viscous cellular DNA. Total cellular protein was used for SDS-polyacrylamide gel electrophoresis. The procedure for immunoblotting with antisera to Ad4BP/SF-1, P450(SCC), and P450(17α) was described previously (*8, 25*). ECL Western blot reagents (Amersham, Arlington Height, IL) were used for the detection. The antiserum to P450(17α) was kindly supplied by Drs. T. Imai (Nagoya Univ.) and M.R. Waterman (Vanderbilt Univ.).

Culture conditions of Y-1 adrenocortical and I-10 testicular Leydig cells were described elsewhere (*26*). For stimulation by cAMP, Y-1 cells were cultured in the presence of 25 μM forskolin for 1, 3, 6, 12, 24, and 48 h, while I-10 cells were cultured in the presence of 1 mM dBtcAMP for 24, 48, 72, 96, and 120 h. The medium containing the stimulant was changed every 24 h. After the incubation, the cells were lysed and used for immunoblotting as described above.

CAT Assay—CAT reporter gene constructs, Ad4ECAT-2.0K and pS2.3H-CAT, were described previously (*27, 28*). In brief, Ad4ECAT2.0K contains a 5.6 kb DNA fragment from 2.0 kb upstream from the transcription initiation site to 5 bp upstream from the initiation methionine in the second exon of the *rFtz-F1* gene in the 5′ upstream region of the *CAT* gene of p00CAT (*27*). pS 2.3H-CAT contains 2.3 kb upstream from the transcription initiation site of the human *CYP11A* gene, which contains proximal and distal promoters. As described previously, the *CYP11A* is transcribed in a cAMP-dependent manner by the function of the cAMP-responsive element (*28*). Y-1 adrenocortical tumor and I-10 testicular Leydig tumor cells were used for the transient transfection assay. Three micrograms of the CAT constructs and 0.2 μg of RSV/luc, which contains a luciferase gene under the control of the Rous sarcoma virus enhancer/promoter, were transfected into the cells by lipofection. After incubation for 36 h in the presence or absence of 25 μM forskolin for Y-1 and 1 mM dBtcAMP for I-10 cells, the cells were harvested and used for CAT assays. The efficiency of transfection into Y-1 cells was normalized by the luciferase activities derived from RSV/luc as described (*4*). In the case of I-10 cells, RSV/luc was not used for normalization since the RSV promoter was activated by the addition of dBtcAMP. Therefore, after transfection of the CAT constructs, the I-10 cells were divided into two aliquots and cultured in the presence or absence of dBtcAMP. CAT assays were performed using 1-deoxy-[dichloro-acetyl-1-^{14}C]chloramphenicol (56 mCi/mmole, Americium, UK). All transfection experiments were performed at least three times.

RESULTS

To assess the effects of the tropic peptide hormones on the *rFtz-F1* gene transcription *in vivo*, the steroidogenic tissues from hypophysectomized rats were investigated. The adrenal gland underwent a striking reduction in volume after the hypophysectomy. The cell number was also reduced significantly in the inner zonae of the cortex, the zona fasciculata and the zona reticularis, whereas that in the outer zone, the zona glomerulosa, seemed to be unchanged. Reduction in cellular volume was another prominent alteration observed especially in the zona fasciculata, which was mainly due to disappearance of lipid droplets, the substrates for steroid synthesis. In spite of these structural alterations induced by hypophysectomy, immunohistochemical analyses revealed that the expression of Ad4BP/SF-1 was maintained in the hypophysectomized rats (Fig. 1). By using the same specimen, the expression of P450(SCC), which catalyzes side-chain cleavage of cholesterol, was also examined. Abundant expression of the P450 was observed in the zonae fasciculata and reticularis of the adrenal cortex of the sham-operated animals, whereas less expression was seen in the zona glomerulosa. In contrast,

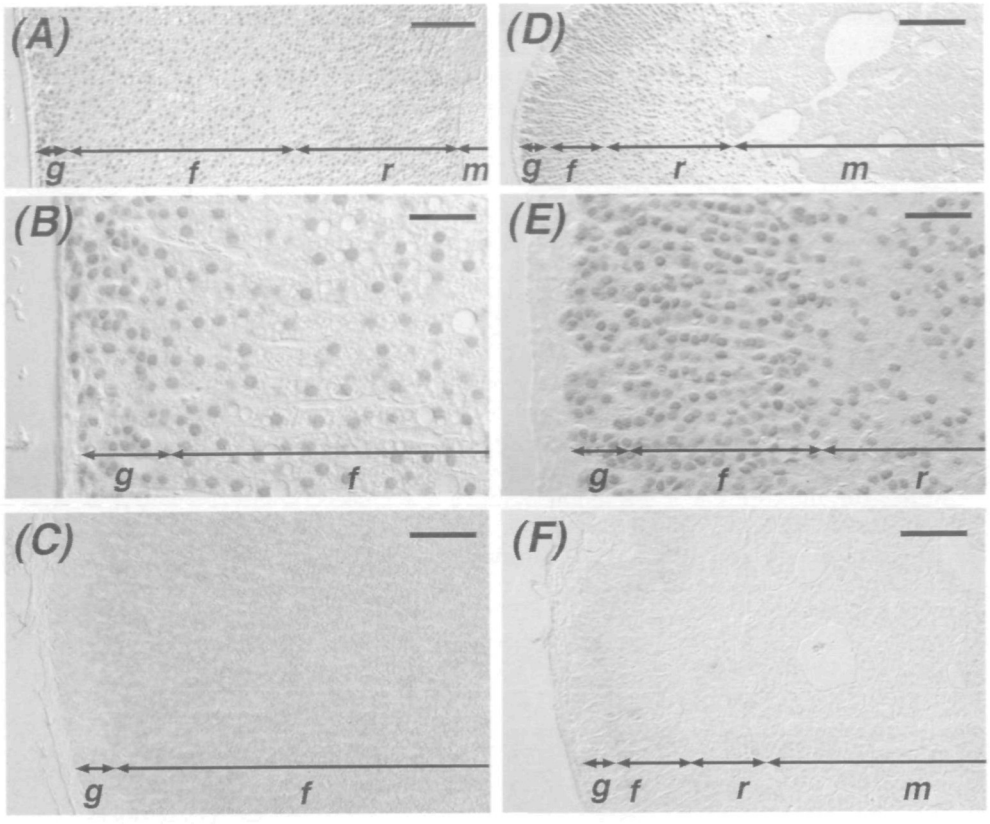

Fig. 1. **Immunohistochemical staining of the adrenal glands from sham-operated and hypophysectomized rats.** The antiserum to Ad4BP/SF-1 was used for staining of sham-operated (A and B) and hypophysectomized (D and E) rat tissues, while the antiserum to P450(SCC) was used for staining of sham-operated (C) and hypophysectomized (F) rat tissues as described in "MATERIALS AND METHODS." Scale bars indicate 200 μm in A and D, 50 μm in B and E, and 100 μm in C and D. g, glomerulosa; f, fasciculata; r, reticularis; m, medulla.

the intensity of staining was reduced in the adrenal cortex of the hypophysectomized animals (data not shown). To confirm the above observations quantitatively, immunoblot analyses were performed with the adrenal cortex isolated under the microscope. Equal amounts of the total cellular proteins prepared from the adrenal cortex of the sham-operated and hypophysectomyzed rats were used for SDS-polyacrylamide gel electrophoresis, followed by immunodetection with the antisera to Ad4BP/SF-1 and P450(SCC). As shown in Fig. 2A, the signals corresponding to Ad4BP/SF-1 were almost equal in the two adrenocortical preparations. On the contrary, the signal of P450(SCC) was reduced considerably by hypophysectomy (Fig. 2B).

The expression of Ad4BP/SF-1 was also investigated with the gonads, testes, and ovaries, of the hypophysectomized animals. Involution was observed clearly in these tissues, as in the adrenal cortex. In the degenerating testes, the diameter of the seminiferous tubules was reduced (Fig. 3, A and B). With respect to spermatogenesis, seminiferous tubules containing mature spermatozoa could not be observed. As described elsewhere (*8, 25*), immunohistochemical analyses detected the expression of Ad4BP/SF-1 in Sertoli and Leydig cells (Fig. 3A). The expression was found to be continued in the same cell types of the hypophysectomized animals (Fig. 3B). For comparison of the amount of Ad4BP/SF-1 expressed in these testes, total tissue lysates were used for immunoblot analyses. As shown in Fig. 2A, a larger amount of Ad4BP/SF-1 was detected in the testes of the hypophysectomized rats than the sham-operated animals. In the testes of the hypophysectomized rats, developing stages of germ cells were observed to decrease significantly. Therefore, protein

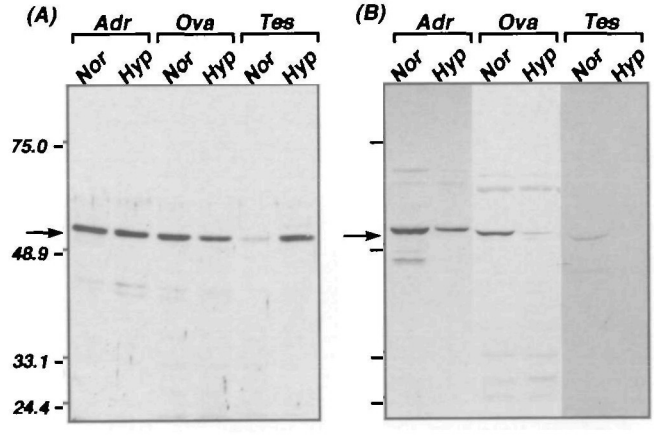

Fig. 2. **Immunoblot analyses of Ad4BP/SF-1 and P450s in sham-operated and hypophysectomized rat tissues.** Total tissue lysates were prepared with the adrenal cortex (Adr) isolated from the adrenal gland under the microscope and whole gonads, testis (Tes) and ovary (Ova). The tissue lysates from sham-operated (Nor) and hypophysectomized (Hyp) animals were subjected to SDS-polyacrylamide gel electrophoresis followed by immunodetection with the antiserum to Ad4BP/SF-1 (A) or P450s, P450(SCC) for the adrenal cortex and ovary or P450(17α) for the testis (B). For detection of Ad4BP/SF-1, 25 μg of protein was used in each lane. For detection of P450(SCC), 25 and 50 μg of proteins were used in the lane of the adrenal cortex and the ovary, respectively. For detection of P450(17α) in the testicular lysates, 100 μg of protein was used. Locations of molecular weight markers are indicated. Arrows indicate Ad4BP/SF-1 in A and P450(SCC) or P450(17α) in B.

components expressed in the somatic cells including Sertoli and Leydig cells should make a larger contribution to the signal intensity of immunoblotting when identical amounts of the proteins from the two testes were used. The stronger signal seems to be mainly due to the secondary effect of alteration in the cellular components of the testes. As the downstream gene of Ad4BP/SF-1 (*29, 30*), a steroidogenic P450, P450(17α), expressed in Leydig cells was examined. The content was decreased significantly by hypophysectomy (Fig. 2B). Unfortunately, no reliable signal was detectable in the testicular preparations in our immunohistochemical study.

Similar alterations were observed with the female gonads. Developing follicles and various stages of corpora lutea were seen in the ovaries of the sham-operated animals. In the hypophysectomized animals, however, only a few preantral follicles surrounded by a small number of theca cells were observed. By contrast, the bulk of the ovary was occupied by corpora lutea. Staining of Ad4BP/SF-1 was observed in the granulosa, theca, and corpus luteum of the sham-operated animals (Fig. 4A) (*8, 24, 25*). In the ovaries of the hypophysectomized animals, the granulosa and theca cells of the preantral follicles were positive for the Ad4BP/SF-1 expression. Ad4BP/SF-1 was also detectable in the corpora lutea (Fig. 4B). The ovaries were used for immunoblot analyses to investigate whether the content of Ad4BP/SF-1 was affected by hypophysectomy. As shown in Fig. 2A, the amounts of Ad4BP/SF-1 seemed to be the same in the two ovaries. Nevertheless, that of P450(SCC) decreased as in the case of the tissues described above.

By using cultured Y-1 and I-10 cells derived from mouse adrenal and testicular Leydig cell tumors, respectively, we examined whether the transcription of the rat *rFtz-F1* gene is activated by cAMP. A CAT reporter gene plasmid, Ad4ECAT2.0K, carrying the 2.0 kb upstream from the transcription initiation site and the first intron of the rat *rFtz-F1* gene, was transfected into Y-1 and I-10 cells. After the cells were cultured in the presence of forskolin or dBtcAMP, the cellular proteins were used for CAT assay. As shown in Fig. 5, the CAT activity driven by Ad4ECAT-2.0K was not induced in Y-1 cells by the treatment with dBtcAMP. Another CAT plasmid, pS2.3H-CAT, carrying the 2.3 kb fragment upstream from the transcription initiation site of the human *CYP11A* gene was also examined under the same conditions. Treatment with the

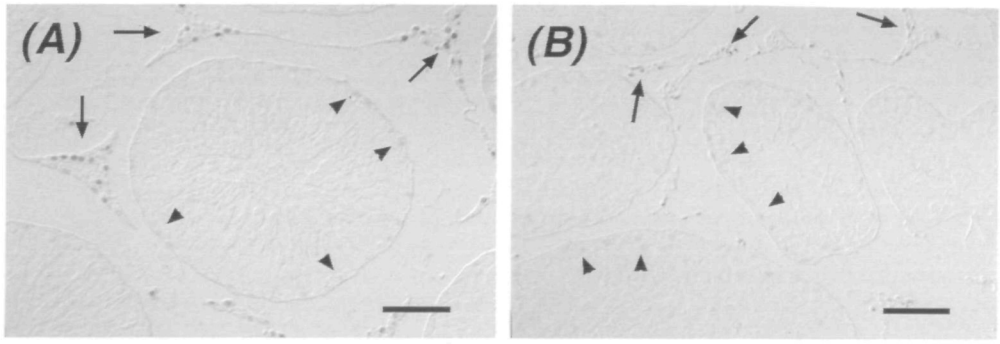

Fig. 3. **Immunohistochemical staining of the testes from sham-operated and hypophysectomized rats.** The antiserum to Ad4BP/SF-1 was used for staining the testes prepared from sham-operated (A) and hypophysectomized (B) animals. Arrows and arrowheads indicate Leydig and Sertoli cells, respectively. Scale bars indicate 100 μm.

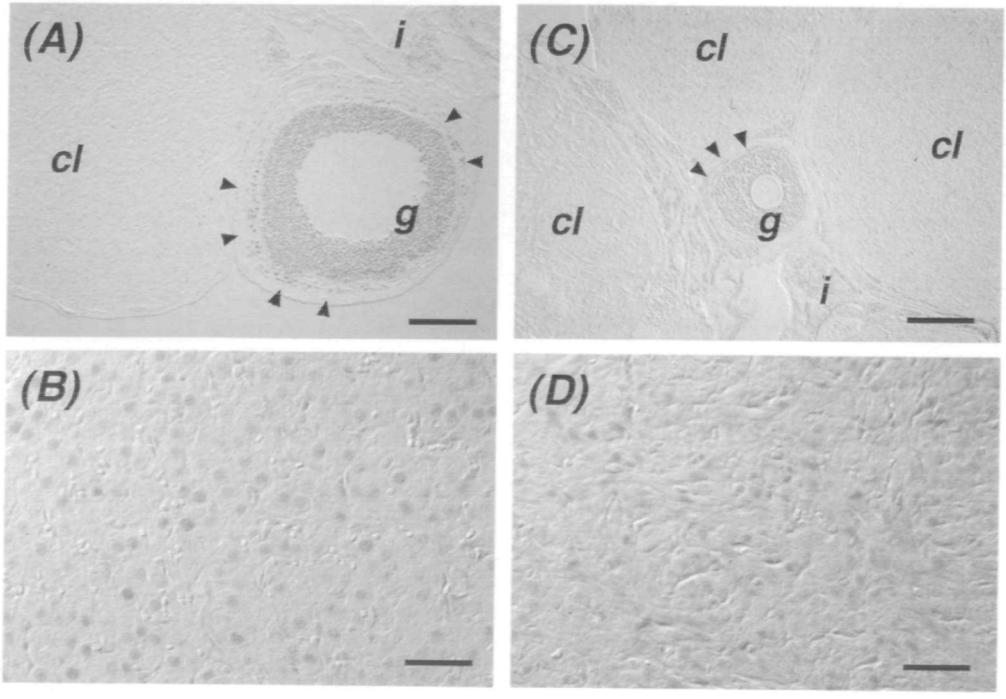

Fig. 4. **Immunohistochemical staining of the ovaries from sham-operated and hypophysectomized rats.** The antiserum to Ad4BP/SF-1 was used for staining the ovaries prepared from the sham-operated (A and B) and hypophysectomized (C and D) animals. B and D show the staining of corpora lutea of the two animals. Arrowheads indicate theca cells. Scale bars indicate 200 μm in A and C, and 50 μm in B and D. g, granulosa; cl, corpus luteum; i, interstitial cells.

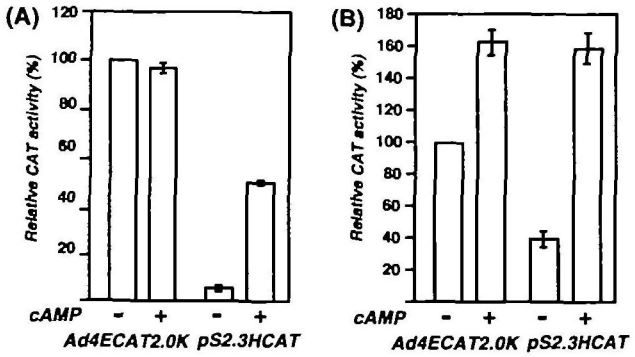

Fig. 5. **Promoter analyses of the *rFtz-F1* and *CYP11A* genes.** The CAT gene constructs with the *rFtz-F1* gene promoter (Ad4ECAT2.0K) and the *CYP11A* gene promoter (pS2.3HCAT) were transfected into (A) Y-1 and (B) I-10 cells followed by incubation in the presence (+) or absence (−) of forskolin and dBtcAMP, respectively. CAT assay was performed as described in "MATERIALS AND METHODS." The CAT activities relative to that of Ad4ECAT2.0K with no effector (−) are shown on the left. Results represent the mean ± SEM for three experiments.

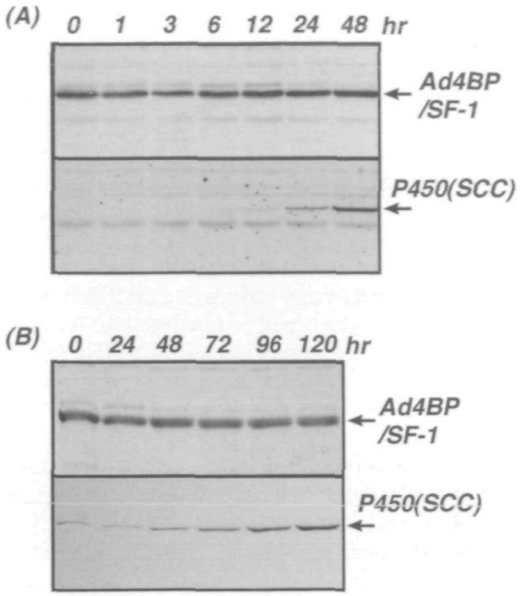

Fig. 6. **Immunoblot analyses of endogenous gene products in Y-1 and I-10 cells.** Y-1 (A) and I-10 (B) cells were cultured in the presence of forskolin and dBtcAMP, respectively, for the indicated time. Total cellular proteins (50 μg) of each time point were used for immunodetection of Ad4BP/SF-1 and P450(SCC). Arrows indicate Ad4BP/SF-1 on the upper and P450(SCC) on the lower panels of each figure.

reagent elevated the CAT activity by approximately 10-fold in Y-1 cells. In I-10 cells, dBtcAMP treatment induced an increase in the CAT activity of pS2.3H-CAT of approximately 4-fold and that of Ad4ECAT2.0K of 1.5-fold.

The effect of cAMP on the endogenous *rFtz-F1* gene transcription of these cells was investigated as follows. Total cellular proteins were prepared from Y-1 and I-10 cells cultured in the presence of forskolin and dBtcAMP, respectively, at the time points indicated in Fig. 6. They were subjected to immunodetection with the antiserum to Ad4BP/SF-1. Approximately identical amounts of Ad4BP/SF-1 were detected regardless of the stimulation both in Y-1 and I-10 cells (Fig. 6). The same protein preparations were then subjected to immunodetection of P450(SCC). Although the P450 was not detected in the untreated Y-1 cells by the immunoblotting, a detectable amount of the P450 accumulated after incubation for 24 h. The amount increased thereafter to 48 h (Fig. 6A). In the I-10 cells, P450(SCC) was detectable even in the absence of the stimulant, whereas accumulation of the protein was not clear until after 24 h of incubation. Thereafter, P450 was accumulated up to 120 h (Fig. 6), in good agreement with the report by Chen *et al.* (*31*). Consequently, the amount of P450(SCC) in both cells was revealed to increase without a concomitant increase in the amount of Ad4BP/SF-1.

DISCUSSION

It has been established that the hypothalamo-pituitary axis is essential for the maintenance of gonadal and adrenocortical functions (*16*). Supporting the function of the axis, hypophysectomy leads to functional defects accompanied by the structural degeneration of these tissues. One of the functional defects is a significant decrease in the steroid hormone synthetic activity, which is caused by the decreased amounts of the steroidogenic P450s (*32-34*). The present study employing immunohistochemical and immunoblot analyses also shows the decreased expression of P450(SCC) in the hypophysectomized rats. Moreover, several studies of the steroidogenic *CYP* gene regulation have indicated that the promoter activities of the genes were enhanced by cAMP, whose intracellular concentration is regulated by the tropic peptide hormones secreted by the pituitary (*26, 28, 35-43*). Therefore, in view of the implication of Ad4BP/SF-1 in the transcription of the steroidogenic *CYP* genes (*1, 44, 45*), it seemed reasonable to hypothesize that the decrease of the Ad4BP/SF-1 content preceded that of the steroidogenic P450s. However, the expression of the transcription factor was found to be maintained even in the absence of any stimulation from the pituitary. In fact, as revealed by the immunoblot analyses, the transcription of the endogenous *rFtz-F1* gene in cultured Y-1 and I-10 cells was not activated by the stimulation of cAMP. Promoter assay using the CAT reporter gene revealed that the *rFtz-F1* gene promoter has no ability to respond to cAMP in Y-1 cells. In I-10 cells, the activity appeared to be enhanced slightly by 1.5-fold. However, because of the constant expression of Ad4BP/SF-1 in the cultured cells, such a small enhancement at the transcriptional level does not seem to result in an increase of the protein. If such an enhancement occurred in the whole animal, therefore it would be unlikely to affect significantly the physiological function of the steroidogenic tissues. As a consequence, the present study with whole animals and cultured cells indicated from physiological aspects that the *rFtz-F1* gene expression is not largely dependent on the pituitary control.

In agreement with our observation, Crawford *et al.* reported that elevated concentration of adrenocorticotropic hormone (ACTH) caused by treatment with LSP (endotoxine) did not lead to an increase in the amount Ad4BP/SF-1 mRNA in the adrenal glands (*46*). In addition, the sup-

pressed expression of 21-hydroxylase P450 by dexamethasone treatment recovered after cessation of the treatment. Under the same conditions, however, the expression of Ad4BP/SF-1 did not recover sufficiently, indicating that an unknown mechanism that is independent of pituitary control probably functions in the *rFtz-F1* gene regulation. In contrast, upregulation of the *rFtz-F1* gene transcription was observed following gonadectomy and administration of gonadotropin-releasing hormone (GnRH) (*47*), although the stimulation by either treatment alone seems weak. Castration and ovarectomy induce 3- and 2-fold activation, respectively, while the GnRH treatment induces 1.5-fold activation. Although the expression of Ad4BP/SF-1 in the pituitary was not investigated in the present study, a distinct mechanism for the gene regulation might function in each tissue.

In addition to these observations, the decrease in the amount of Ad4BP/SF-1 was indicated in degenerating corpora lutea and atretic follicles in normally cycling ovaries (*24*). Because regression of the corpus luteum and formation of the follicular atresia are accompanied by reduction of steroid hormone synthetic activity resulting from disappearance of the steroidogenic P450s, it is reasonable to assume that the *rFtz-F1* gene can be downregulated in certain situations. Although the mechanism of this downregulation is unclear, apoptotic cell death might be a closely related event. Supporting such a correlation, apoptotic cell death was reported to occur in the atretic follicles and degenerating corpora lutea (*48–50*). The onset of apoptotic cell death might induce inactivation of the *rFtz-F1* gene transcription, or alternatively the inactivation might be an essential step for the apoptotic cell death in the steroidogenic cells.

The above observations, including those of the present study, strongly suggest that the induction of the steroidogenic *CYP* gene transcription by cAMP is not caused by an increase in the amount of Ad4BP/SF-1. With respect to the mechanisms underlying transcription of certain genes, several events such as phosphorylation (*51*), ligand binding (*52, 53*), and proteolysis (*54*) have been reported to control the transcriptional efficiency, probably through modifying interactions with other factors. Phosphorylation and ligand binding should be considered in the case of Ad4BP/SF-1-dependent transcriptional regulation of the steroidogenic *CYP* genes. As described elsewhere (*55*), phosphorylation sites of protein kinase A (PKA) and protein kinase C were found in the primary structure of Ad4BP/SF-1. Indeed, *in vitro* phosphorylation of Ad4BP/SF-1 by PKA was shown recently (*30*). The phosphorylation of Ad4BP/SF-1 by PKA might participate in the transcriptional activation of the steroidogenic *CYP* genes by the tropic peptide hormones, although the precise mechanism remains to be elucidated. Regarding the ligand molecule, a recent study by Lala *et al.* (*56*) showed that oxycholesterols function as the ligands for Ad4BP/SF-1. Since ligand molecules control the function of the hormone receptors (*52*), the concentration and distribution of the oxycholesterols in particular situations should be considered in order to understand the mechanisms regulating the downstream genes.

Ad4BP/SF-1 is essential for the maintenance of the functions of the steroidogenic tissues. Moreover, disruption studies clearly indicated that the *rFtz-F1* gene plays critical roles in gonadal and adrenal differentiation (*11, 20, 21*). From both aspects of the functional significance of Ad4BP/SF-1, it is easily understood that disappearance of the transcription factor probably leads to severe functional defects of the tissues. Consequently, the *rFtz-F1* gene might continue to be expressed after the onset of the transcription, with some exceptions in ovarian cells. Therefore, it is probable that the expression of Ad4BP/SF-1 is independent of the pituitary stimulation, while the transcription of the downstream genes is strictly under the regulation of this stimulation.

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