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Gene Regulation of Steroidogenesis

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The biosynthesis of various steroid hormones in animal tissues are catalyzed by six forms of cytochrome *P*450 and two hydroxysteroid dehydrogenases. The tissue-specific expression of these enzymes, which are under the control of the pituitary gland and mainly regulated at the transcriptional level, determines the steroidogenesis of animal tissues. Analysis of the promoter regions of the steroidogenic *P*450 genes revealed various *cis*-acting elements, including cAMP-responsive sequences (CRS), Ad4, and GC-rich sequences, which were needed for the tissue-specific and cAMP-dependent expression of the genes. Some of the nuclear protein factors binding to the *cis*-acting elements were isolated and characterized. A zinc-finger protein binding to Ad4, which was termed Ad4BP or SF-1, seems to be of particular importance in steroidogenesis. Ad4BP was expressed in the cells of the steroidogenic tissues, adrenal gland and gonadal tissues, in the rat fetus prior to the expression of steroidogenic *P*450s, and remained expressed only in steroidogenic cells in adult animals. Close investigation of the temporal and spacial expression of Ad4BP in the fetal tissues suggested its role in the differentiation of the steroidogenic tissues and the sex determination of the gonadal tissues.

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INTRODUCTION

Six forms of cytochrome *P*450 and two hydroxysteroid dehydrogenases are the main steroidogenic enzymes involved in the biosynthesis of various steroid hormones starting from cholesterol in animal tissues (Fig. 1), and the tissue-specific expression of a combination of these enzymes, particularly of the *P*450s, determines the end products of the synthetic pathway in each tissue. Three of the six steroidogenic *P*450s, $P450_{scc}$, $P450_{11\beta}$, and $P450_{aldo}$, are located in the inner membrane of mitochondria, whereas the other three, $P450_{c21}$, $P450_{17\alpha}$, and $P450_{arom}$, are in the membrane of endoplasmic reticulum. The two hydroxysteroid dehydrogenases, 3β-HSD and 17β-HSD, are also associated with the endoplasmic reticulum membrane.

The cleavage of the side chain of cholesterol by $P450_{scc}$ to form pregnenolone is the initial reaction as well as the rate-limiting step of the steroid hormone biosynthesis. Acute stimulation of steroid hormone production by the pituitary hormones depends exclusively on enhanced availability of cholesterol for the side chain cleavage reaction, not on the change in the amount of $P450_{scc}$. Slower stimulation involves an increase in the amounts of the steroidogenic enzymes including $P450_{scc}$, and the increase is mainly due to increased transcription of the genes of the enzymes. Stational level expression of the enzymes in the steroidogenic tissues is also dependent on continuous stimulation by the peptide hormones from the pituitary gland. Available evidence indicates that cAMP is the main mediator in the hormonal induction of the steroidogenic enzymes in the cells of adrenocortical and gonadal tissues.

Analyses of the promoter regions of the steroidogenic *P*450 genes have been carried out in the past years to elucidate the mechanism of tissue-specific and cAMP-regulated expression of the genes in the steroidogenic tissues. The cAMP-responsive sequence (CRS) has been identified in the promoter region for each *P*450 gene [1], but no common sequence has been identified among the CRSs, although the transcription of the *P*450 genes in the steroidogenic tissues are coordinately activated by the pituitary hormones. Various *cis*-acting elements have also been shown to be involved in the tissue-specific expression of the steroidogenic *P*450s, and several transcription factors have been characterized. Among the proposed transcription factors, Ad4BP [2] (or SF-1 [3]) seems to be of particular

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importance not only for the expression of steroidogenic enzymes but also for the development and differentiation of the steroidogenic tissues in the fetal and postnatal animals.

PARTICIPATION OF BOTH MITOCHONDRIAL AND MICROSOMAL *P*450s IN STEROID HORMONE BIOSYNTHESIS

One characteristic feature of steroid hormone biosynthesis in animal tissues is the participation of both mitochondrial and microsomal *P*450s. $P450_{scc}$, which catalyzes the initial step of steroid hormone biosynthesis, is a mitochondrial *P*450. Since the substrate of the reaction, cholesterol, is synthesized in the cytoplasm and stored in cytoplasmic oil droplets in an esterified form, it must be transported from the cytosol into the mitochondria to be metabolized by $P450_{scc}$ in the inner membrane, and the product of the reaction, pregnenolone, is transported out of the mitochondria into the cytosol to be further metabolized by microsomal HSDs and *P*450s. In the case of adrenal cortex cells, the final steps of steroid hormone biosynthesis to form glucocorticoids and mineralcorticoids are catalyzed by two mitochondrial *P*450s, $P450_{11\beta}$ and $P450_{aldo}$. Thus the intermediates of the steroid hormone biosynthesis shuttle between cytosol and mitochondria across the mitochondrial membranes.

The mitochondrial *P*450s commonly require two electron-transfer components in the matrix, adrenodoxin and NADPH-adrenodoxin reductase, for the supply of electrons from NADPH to catalyze the oxygenation reactions. These two electron-transfer components are soluble matrix proteins, but they seem to be loosely associated with the membrane-bound *P*450s in mitochondria. The microsomal *P*450s depend on a membrane-bound flavoprotein, NADPH-cytochrome *P*450 reductase, for the supply of electrons. It is also known that cytochrome b_5 in the same membrane interacts with the *P*450s and modulates their oxygenase activities. When the steroidogenic cells are stimulated by pituitary peptide hormones, both mitochondrial and microsomal steroidogenic *P*450s increase. Therefore, we can expect to find a common regulatory mechanism for the expression of all steroidogenic *P*450s in steroid hormone-producing tissues.

GENES OF STEROIDOGENIC *P*450s

Although the major biosynthetic pathways of the steroid hormones from cholesterol in animal tissues had been elucidated many years ago, the purification and characterization of the steroidogenic *P*450s catalyzing the oxygenation reactions in the biosynthetic pathways was not completed until recently. The last one of the steroidogenic *P*450s to be characterized was $P450_{aldo}$, which was purified as late as in 1989 [4], and whose gene was characterized only a few years ago.

The structures of the genes of the six steroidogenic *P*450s have been elucidated. Their exon–intron structures are schematically shown in Fig. 2. The genes of the three mitochondrial *P*450s, $P450_{scc}$ gene (CYP11A), $P450_{11\beta}$ gene (CYP11B1), and $P450_{aldo}$ gene (CYP11B2), have the same number of introns inserted at the corresponding positions in the coding sequences, whereas the exon–intron structures of the genes of the three microsomal *P*450s, $P450_{c21}$ gene (CYP21B), $P450_{17\alpha}$ gene (CYP17), and $P450_{arom}$ gene (CYP19), are apparently different from each other.

Expression of these *P*450 genes is steroidogenic tissue-specific, hormonally controlled via intracellular cAMP concentrations, and regulated during the

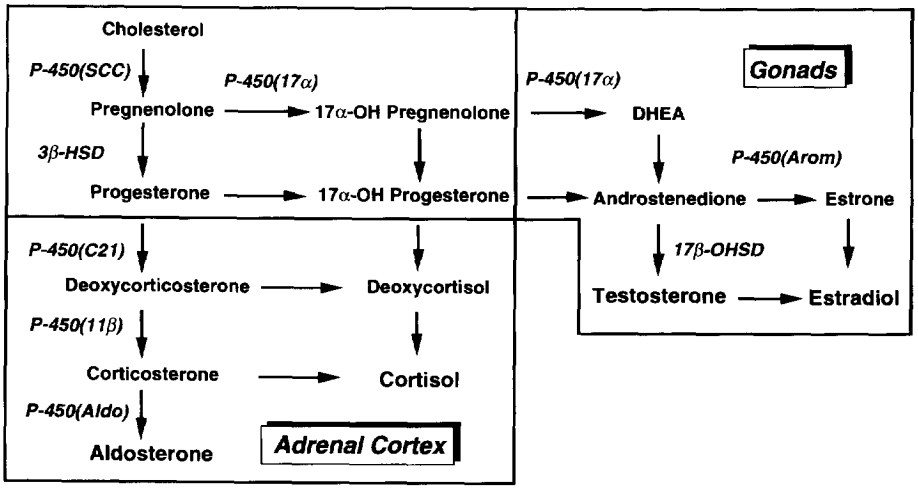

Fig. 1. Biosynthesis of steroid hormones from cholesterol in adrenal cortex and gonadal tissues. Participations of six forms of cytochrome *P*450 and two hydroxysteroid dehydrogenases in each step of the synthetic pathways are indicated in the figure.

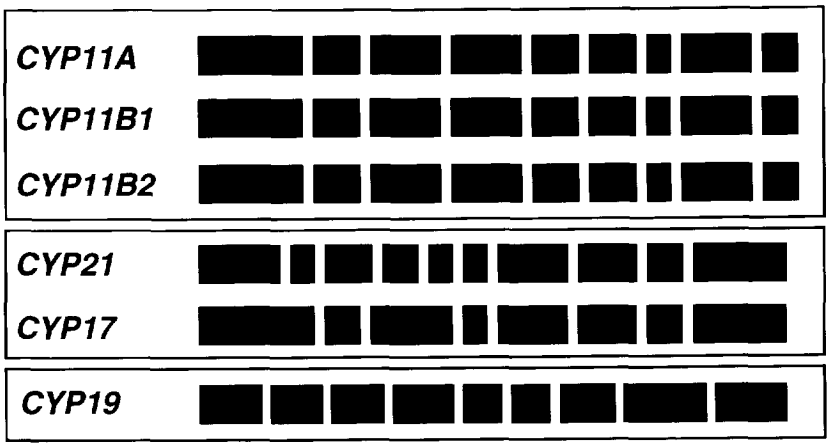

Fig. 2. Exon–intron structures of steroidogenic *P*450s. The exons of each gene are schematically shown by the shadowed boxes. The positions of intron insertion are indicated by the gaps between the exons.

development of the steroidogenic tissues in fetal and postnatal animals. Expression of the genes of the steroidogenic *P*450s, which determines the steroidogenesis of the animal tissues, is not only specific for each tissue, but also cell type-specific in the tissue (Fig. 3). In the adrenal cortex of the rat, mouse, and human, $P450_{aldo}$ is expressed only in the cells of the zona glomerulosa, which correlates well with the production of aldosterone in the zona glomerulosa, and not in the zona fasciculata nor in the zona reticularis. Since steroid hormones are not stored in the steroid hormone producing cells, the secretion of the steroid hormones from the cells correlates directly with the activities of the steroidogenic enzymes in the cells. The molecular mechanism of the tissue-specific and pituitary hormone-regulated expression of the *P*450 genes in the steroidogenic tissues is the central problem of steroidogenesis.

ADRENAL CORTEX-SPECIFIC AND cAMP-REGULATED EXPRESSION OF $P450_{11\beta}$ GENE

$P450_{c21}$, $P450_{11\beta}$ and $P450_{aldo}$ are expressed only in adrenal cortex and the latter two catalyze the final steps of corticosteroids biosynthesis. To study the adrenal cortex-specific and cAMP-regulated expression of these genes, we first analyzed the promoter region of a bovine $P450_{11\beta}$ gene by *in vitro* transcriptions with adrenal cortex nuclear extracts, by CAT assays with cultured steroidogenic cells, and by foot printing with adrenal cortex nuclear extracts, and found six *cis*-acting elements, Ad1, Ad2, Ad3, Ad4, Ad5, and Ad6, in the 5′-upstream region of the gene [5, 6] (Fig. 4). When the nucleotide sequences of these *cis*-acting elements were compared with the corresponding regions of mouse, rat, and human $P450_{11\beta}$ genes, they were highly conserved among the animal species.

	P-450(SCC)	P-450(11β)	P-450(Aldo)	P-450(C21)	P-450(17α)	P-450(Arom)	Hormone
Adrenal Cortex							
Z. glomerulosa	+	−	+	+	−	−	*Aldosterone*
Z. fasciculata	+	+	−	+	+	−	*Cortisol*
Z. reticularis	+	+	−	+	+	−	*Cortisol*
Testis							
Sertoli	−	−	−	−	−	+/−	*(Estrodiol)*
Leydig	+	−	−	−	+	−	*Testosterone*
Ovary							
Glanulosa	+	−	−	−	−	+	*Testosterone*
Theca	+	−	−	−	+	−	*Estradiol*
Corpus Luteum	+	−	−	−	−	+/−	*Progesterone*

Fig. 3. Cell type-specific expression of steroidogenic *P*450s in the cells of adrenal cortex and gonadal tissues. The types of the cells in each tissue are shown on the left, and the major steroid hormones produced by the cells are indicated on the right. Plus and minus symbols indicate the expression and non-expression of each *P*450, respectively.

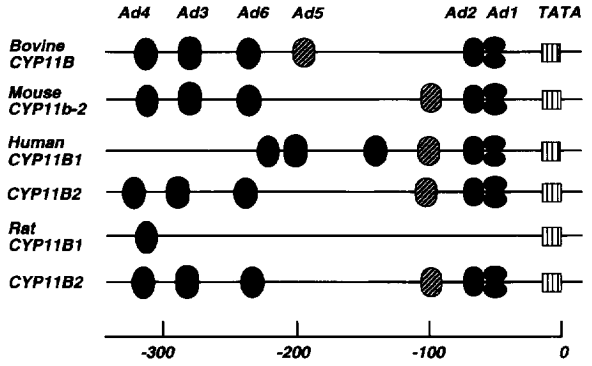

Fig. 4. *cis*-Acting elements in the promoter regions of CYP11 genes. The positions of the TATA box and the *cis*-acting elements, Ad1 ~ Ad6, in the promoter regions of $P450_{11\beta}$ and $P450_{aldo}$ genes are indicated by the binding factors shown by ovals. The distance from the transcription start site is indicated by the number in base pairs.

Ad1 and Ad2 were needed for the *in vitro* transcription of bovine $P450_{11\beta}$ gene with bovine adrenal cortex nuclear extract [6]. Ad1 is apparently a cAMP-responsive element where CREB binds, but the response of $P450_{11\beta}$ gene to cAMP requires Ad3 and Ad4 in addition to Ad1 [5].

Gel-shift assays with the nuclear extracts prepared from rat tissues revealed the presence of protein factors binding to the *cis*-acting elements in the 5′-upstream region of the $P450_{11\beta}$ gene. Interestingly, the protein factor binding to the Ad4 site was present only in the adrenal nuclear extract, whereas the protein factors binding to the other five sites were present not only in the adrenal gland but also in other non-steroidogenic tissues including liver, spleen, and brain. The Ad4 site-binding protein was named Ad4BP [2].

STEROIDOGENIC CELL-SPECIFIC TRANSCRIPTION FACTOR Ad4BP (SF-1) WHICH REGULATES THE EXPRESSION OF ALL STEROIDOGENIC *P*450 GENES

Ad4BP was purified from bovine adrenal cortex nuclear extract as a 53 kDa protein, and specific binding of Ad4BP to the Ad4 site of the $P450_{11\beta}$ gene was confirmed with the purified preparations [2]. The binding sequence for Ad4BP was analyzed by gel shift assays with synthetic oligonucleotides, and the strong binding sequences were PyCAAGGPyPyPu. Weaker binding was observed with the sequences PuPuAGGTCA. When the 5′-upstream regions of the genes of other steroidogenic *P*450s were searched for potential binding sites of Ad4BP, binding sequences were found with all the steroidogenic *P*450 genes [2], some of which, including the $P450_{11\beta}$ gene, had multiple Ad4BP-binding sequences (Fig. 5), whereas most of non-steroidogenic *P*450 genes lacked Ad4 site. Moreover, in addition to adrenal gland and adrenal-derived Y-1 cells, the expression of Ad4BP was confirmed by Western blotting for all the other steroidogenic cells examined including ovarian granulosa cells and testicular Leydig cell-derived I-10 cells. These observations supported the view that Ad4BP is an essential transcription factor for the tissue-specific expression of the steroidogenic *P*450s. An interesting case is the two isozymes of 3β-HSD, 3β-HSD-I and 3β-HSD-II, whose genes are highly homologous with each other. The former isozyme has no Ad4 site in the 5′-upstream region of the gene and is expressed widely among various tissues, while the latter has a Ad4 site and is expressed only in steroidogenic tissues, suggesting a role of Ad4BP in the tissue-specific expression of 3β-HSD-II.

The amino acid sequence of bovine Ad4BP was deduced from the nucleotide sequence of the cloned cDNA [7]. Ad4BP consisted of 461 amino acids, and its calculated molecular weight was 51 kDa, which was in good agreement with the molecular weight of the purified Ad4BP determined by SDS–PAGE. Comparison of the amino acid sequence with other known transcription factors revealed that Ad4BP is a novel member of the steroid hormone/thyroid hormone receptor superfamily, having a zinc finger domain and a putative ligand binding/dimerization domain. Ad4BP showed high homology with FTZ-F1, which regulates the *fushi tarazu* gene of *Drosophila*, and also with ELP, which is a mammalian homologue of FTZ-F1 and is expressed in murine embryonic carcinoma cells (Fig. 6). The homologies of the zinc finger domain of Ad4BP with those of FTZ-F1 and ELP were 85 and 100%, respectively, whereas the homology with other known members of the steroid hormone/thyroid hormone receptor superfamily was 50–60%. The mouse counterpart of Ad4BP was independently identified as the key regulator of steroidogenic enzyme expression by Parker and his collaborators, and named "steroidogenic factor 1" or SF-1 [3]. The analysis of rat Ad4BP gene revealed that Ad4BP and ELP are tran-

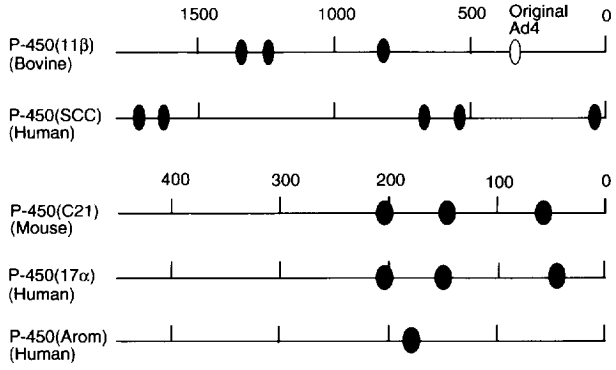

Fig. 5. Ad4 sites in the 5′-upstream regions of the genes of steroidogenic *P*450s. The animal species is indicated for each gene. The positions of the Ad4 sites in the 5′-upstream regions of the genes are shown by closed ovals which represent the binding protein, Ad4BP. The Ad4 site first found in the bovine $P450_{11\beta}$ gene is shown by an open oval.

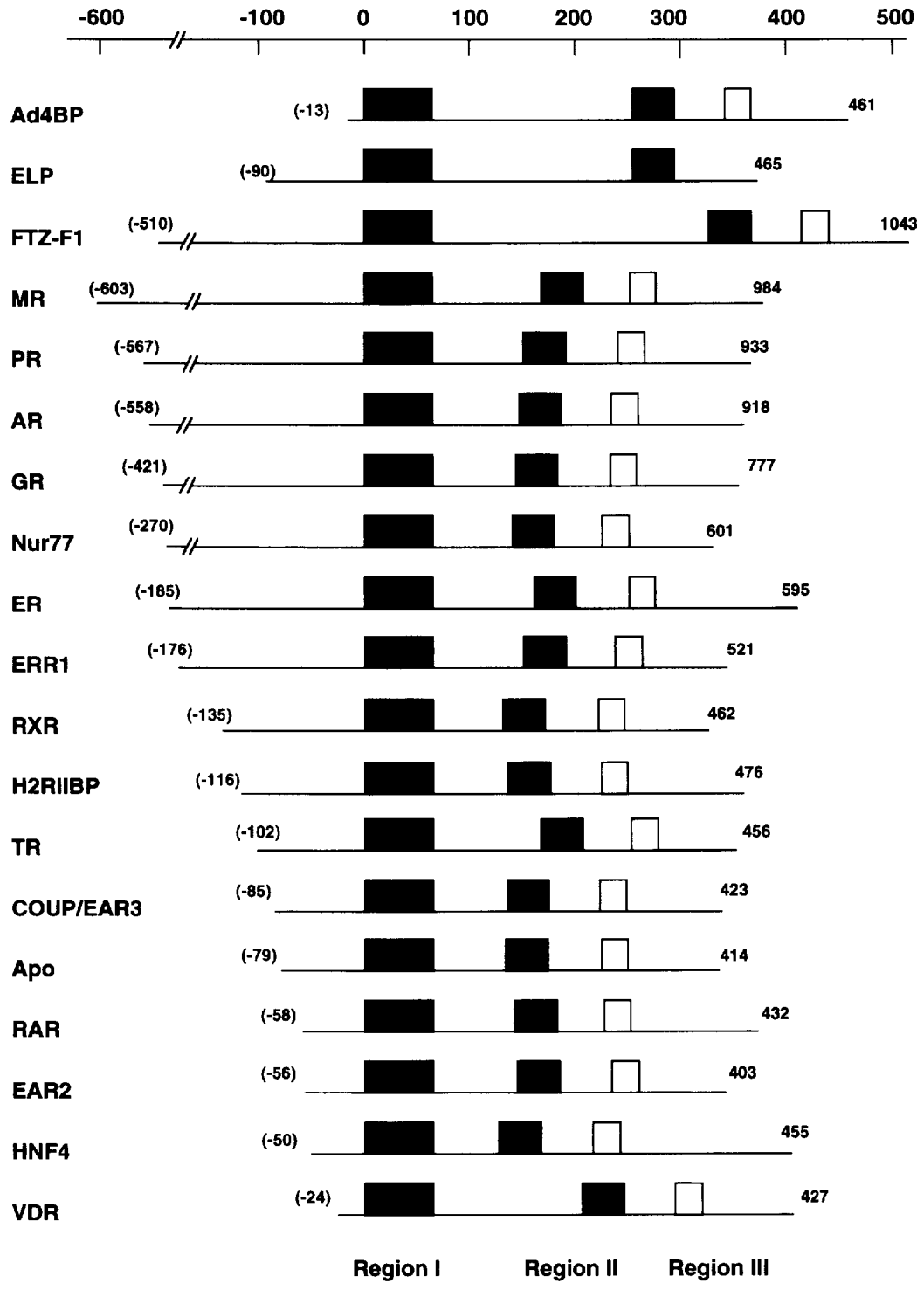

Fig. 6. Schematic comparison of the structure of Ad4BP with the members of steroid hormone/thyroid hormone receptor superfamily [7]. The closed, shaded, and open squares indicate the regions I, II, and III, respectively. The number in the parenthesis on the left for each receptor indicates the number of the amino acids preceding the region I. The number on the right indicates the total number of amino acids of each receptor. The scale of the amino acid sequences is shown above by the number of amino acids.

scribed from a single gene by the usage of different promoters and also by alternative usage of the splice sites. It was found that ELP was also expressed in rat adrenal gland and gonads, but its expression level and its affinity for Ad4 site were much lower than Ad4BP [8]. ELP does not seem to play an important role in the expression of steroidogenic *P*450s in the adrenal gland and gonads.

The essential role of Ad4BP in the expression of steroidogenic *P*450 genes was confirmed by expressing Ad4BP in cultured non-steroidogenic cells which otherwise do not activate steroidogenic *P*450 genes. When the 5′-upstream region of bovine $P450_{11\beta}$ gene was connected to CAT gene and introduced into cultured cells, the steroidogenic Y-1 cells and I-10 cells expressed CAT activity, whereas the non-steroidogenic CV-1 cells did not transcribe the introduced plasmid. However, co-transfection of Ad4BP expression vector together with cAMP-dependent protein kinase (PKA) expression vector enabled the CV-1 cells to transcribe the introduced CAT construct (Fig. 7). The same result was obtained with a CAT gene construct with the 5′-upstream region of human $P450_{scc}$ gene [9]. Y-1 cells and I-10 cells contained endogenous Ad4BP, but the expression of Ad4BP was not detectable in the original CV-1 cells by Western blot analysis with Ad4BP antibody. The non-steroidogenic CV-1 cells were thus converted to steroidogenic cells by the expression of Ad4BP. This observation confirmed the principal role of Ad4BP in tissue-specific expression of steroidogenic enzymes.

In situ hybridization [10] and immunohistochemical staining [8] of the adrenal glands, testes, and ovaries of adult rats or mice also produced strong supporting evidence for the role of Ad4BP in steroidogenesis. The cells which were shown to be expressing Ad4BP coincided with the known steroid hormone-producing cells in the tissues [8], adrenocortical cells in the adrenal gland, Leydig cells in the testis, and granulosa cells and theca cells in the ovary. Judging from the presence of the Ad4 site in the 5′-upstream regions of all the steroidogenic *P*450s, Ad4BP seems to be a common transcription factor regulating the expression of all steroidogenic enzymes including the steroidogenic *P*450s.

EXPRESSION OF Ad4BP IN ADRENAL GLAND AND GONADS IN THE DEVELOPMENTAL STAGES OF FETAL RATS

Immunohistochemical staining of the sections of fetal rats with Ad4BP antibody showed intense staining of the cells of the adrenal gland and gonads at various developmental stages of the fetus [11]. The staining was clearly detected in the primordial adrenal gland and gonads of the fetal animals at 13.5 days postcoitum (d.p.c.), when the sexual differentiation of the gonadal tissue into testis and ovary was morphologically detectable but the steroidogenic enzyme $P450_{scc}$ was not yet expressed as judged by Western blotting of the tissues. In the gonads of the fetus at 14.5 d.p.c., the expression level of Ad4BP in the somatic cells of the testis was much higher than in the ovary, and the sexually dimorphic expression of Ad4BP continued throughout the fetal and neonatal ages of the animals, which suggested its role in the sexual differentiation of the gonadal tissues. The expression of the steroidogenic *P*450s was detected in the adrenal gland and also in the gonads of the 14.5 d.p.c. fetus.

Sexually dimorphic expression of Ad4BP observed in the gonadal tissue in the developmental stage of rat fetus just after sex differentiation seems to suggest that the Ad4BP gene is one of the target genes of the testis-determining factor encoded by the SRY gene, which triggers a cascade of gene expressions leading to the sex differentiation of the fetal animals. It has recently been reported [12] that the gene of Müllerian inhibiting substance (MIS) is regulated by SF-1 (Ad4BP), which confirms the role of Ad4BP as a link between SRY gene and MIS gene in the sex determination cascade of gene expressions. Comparison of the 5′-upstream regions of human, bovine, rat, and mouse MIS genes showed a highly conserved common Ad4 site at around −60 bp [11].

Ad4BP AS AN ESSENTIAL FACTOR IN GENE REGULATION FOR STEROIDOGENIC TISSUE DEVELOPMENT AND SEXUAL DIFFERENTIATION

All of the available evidence indicates the principal role of Ad4BP in the expression and regulation of the genes of steroidogenic *P*450s, and possibly of other steroidogenic enzymes too, in animal tissues. Regulation of the MIS gene by Ad4BP has confirmed the

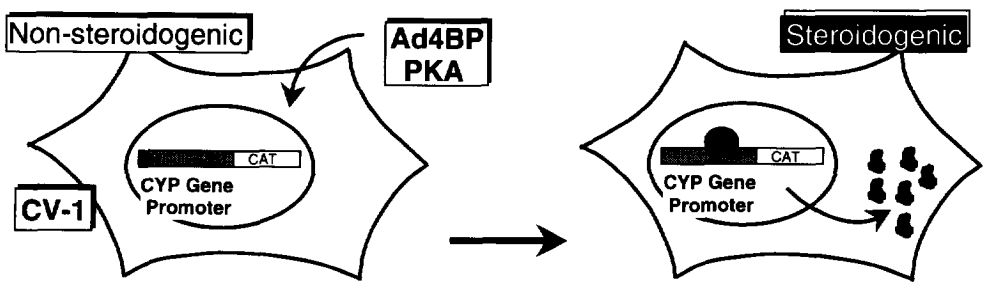

Fig. 7. Transcription activation of steroidogenic *P*450 genes by Ad4BP. The conversion of the non-steroidogenic CV-1 cells to the steroidogenic by the expression of Ad4BP is schematically shown. A CAT construct with the 5′-upstream region of $P450_{11\beta}$ gene was expressed in CV-1 cells when an Ad4BP expression vector and a cAMP-dependent protein kinase (PKA) expression vector were cotransfected.

role of Ad4BP in the sexual differentiation of the gonadal tissues in the fetal animals. A recent report by Luo *et al.* [13] indicated another important function of Ad4BP during the embryonic development of mice. They carried out targeted disruption of the FTZ-F1 gene (Ad4BP gene) with mice, and found that the FTZ-F1 null animals were born alive without the adrenal gland and gonads, indicating the decisive role of the gene in the genesis of the steroidogenic tissues during the embryonic development of the animals.

Ad4BP is an essential factor not only for the expression and regulation of steroidogenic enzymes in animal tissues, but also for the formation of steroidogenic tissues in embryonic animals and the sexual differentiation of the gonads in fetal animals. Elucidation of the mechanism of regulation of the Ad4BP gene during the embryonic and fetal periods of animals will be the next important problem to be clarified.

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