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Function and Distribution of a Steroidogenic Cell-specific Transcription Factor, Ad4BP

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Ad4BP was identified as an essential transcription factor regulating steroidogenic cell-specific and cAMP-dependent transcription of the genes of steroidogenic *P*450s. The Ad4BP transcript was detected in steroidogenic tissues such as adrenal gland, testis, ovary, placenta and brain by RT-PCR, and showed good correlation with the expression of steroidogenic *P*450s. The genes of steroidogenic *P*450s, which are transcribed only in steroidogenic cells, were transcribed in non-steroidogenic cells when an Ad4BP expression vector was introduced into the cells. To study the function of Ad4BP in the differentiation of the steroidogenic tissues, immunochemical and immunohistochemical studies were performed with the tissues prepared from various developmental stages of rats. Adrenal cortex expressed Ad4BP since the tissue was detected in the dorsal wall of the fetus. Gonadal tissues expressed Ad4BP in a sex-dependent manner. High levels of Ad4BP expression were detected in fetal and prepubertal testes and in prepubertal and adult ovaries, whereas low level expressions were observed in the adult testes and in the fetal ovaries. The expression of Ad4BP in the gonads correlates well with the expression of the Müllerian inhibiting substance gene as well as the steroidogenic *P*450 gene for both sexes. These observations indicate that Ad4BP plays an important role in the development and differentiation of the steroidogenic tissues including sexual differentiation of the gonadal tissues through activation of the transcription of its target genes.

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INTRODUCTION

Six forms of cytochrome *P*450 participate in the biosynthesis of steroid hormones from cholesterol in the steroidogenic tissues, and the combinations of the steroidogenic *P*450s expressed in the tissues determine the steroid hormones produced. We studied the tissue-specific and cAMP-dependent expression of one of the steroidogenic *P*450s, $P450_{11\beta}$, in adrenocortical cells, and identified several *cis*-acting elements, Ad1–Ad7, in the promoter region of the bovine $P450_{11\beta}$ gene [1–3]. Nuclear proteins which bind to the *cis*-acting elements were examined by gel shift assays, and we focused our attention on a nuclear protein which binds to the Ad4 site of the gene. The Ad4-binding protein, which was termed Ad4BP, was detected in the nuclear extracts of all the steroidogenic tissues examined including adrenal cortex, but was not present in non-steroidogenic tissues [4]. Ad4BP was purified from the nuclear extracts of bovine adrenal cortex, and its binding nucleotide sequences were determined by the use of the purified preparations; the strong binding sequences were PyCAAGGPyPyPu, and weaker binding was observed with the sequences PuPuAGGTCA [4]. The mouse counterpart of Ad4BP was independently identified as the transcription factor of steroidogenic enzymes by Parker and his collaborators, and they named it SF-1 [5].

The amino acid sequence of bovine Ad4BP determined by cDNA cloning revealed that Ad4BP is a novel member of the steroid hormone/thyroid hormone receptor superfamily [6], and highly homologous to FTZ-F1, which regulates the *fushi tarazu* gene during the embryonic development of *Drosophila*, and also to ELP, which is a mammalian homolog of FTZ-F1 and is expressed in the murine embryonal carcinoma cells. The function of Ad4BP as the steroidogenic cell-specific transcriptional factor was confirmed by the transfection of an Ad4BP expression vector into non-steroidogenic cultured cells, which resulted in the

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transcription of the reporter genes carrying the Ad4 sequence in the promoter regions [7].

Immunohistochemical studies on the expression of Ad4BP in the adrenal gland and gonads of adult rats confirmed its function in steroidogenesis. The expression of Ad4BP in those tissues was also studied at various developmental stages of fetal and postnatal animals, and gave evidence for its role in the development and differentiation of steroidogenic tissues and in the sexual differentiation of gonadal tissues.

REGULATION OF TISSUE-SPECIFIC EXPRESSION OF STEROIDOGENIC *P*450 GENES BY Ad4BP

The *cis*-acting transcription element Ad4, which was originally identified in the promoter region of the $P450_{11\beta}$ gene as an element needed for the cAMP-dependent expression of the gene, is present in the 5′-upstream regions of the genes of all the other five steroidogenic *P*450s, $P450_{scc}$, $P450_{aldo}$, $P450_{C21}$, $P450_{17\alpha}$, and $P450_{arom}$ [4]. Moreover, the nuclear protein binding to the Ad4 site, Ad4BP, is expressed only in the steroidogenic tissues, and not in other non-steroidogenic tissues [4]. These lines of evidence strongly suggested a role of Ad4BP in tissue-specific expression of the steroidogenic *P*450 genes in addition to its function in the cAMP-regulated expression of the genes.

The transcription activity of Ad4 and its activation by Ad4BP were confirmed by the transfection of an Ad4BP expression vector into non-steroidogenic cultured cells, PC-12 cells and CV-1 cells, which are devoid of endogenous Ad4BP [7]. Figure 1 shows the results of a transfection experiment with CV-1 cells.

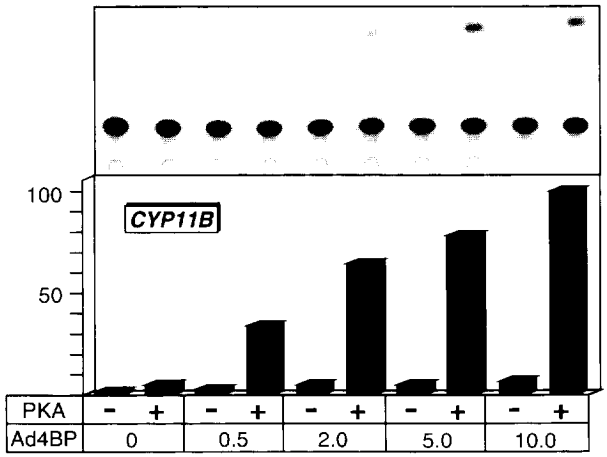

Fig. 1. Transcriptional activation of $P450_{11\beta}$ (CYP11B) gene by Ad4BP [7]. The 1.5 kb 5′-upstream region of bovine $P450_{11\beta}$ gene was connected to the CAT reporter gene. Five μg of the CAT construct was transfected into CV-1 cells with increasing amounts (0–10 μg) of the Ad4BP expression vector in the presence (+) and absence (−) of the PKA expression vector. The relative CAT activities of the transfected CV-1 cells are shown on the lower panel.

The 1.5 kb 5′-upstream region of the bovine $P450_{11\beta}$ gene, which contained four Ad4 sites [3], was connected to the CAT reporter gene. When the CAT construct was introduced into cultured cells, CAT activity was expressed in the steroidogenic Y-1 cells, but not in the non-steroidogenic CV-1 cells. However, co-transfection of an Ad4BP expression vector and a cAMP-dependent protein kinase (PKA) expression vector with the CAT construct resulted in good expression of CAT activity in the CV-1 cells. The expression was dependent on Ad4BP, and stimulated by PKA. Similar results were obtained when the 2.3 kb 5′-upstream region of the human $P450_{scc}$, which contained five Ad4 sites, was connected to the CAT reporter gene and introduced into steroidogenic and non-steroidogenic cultured cells. In the case of the $P450_{scc}$ gene, a distal promoter located at −1.8 to −1.5 kb was necessary for the transcriptional activation of the gene by cAMP, and the distal promoter contained two Ad4 sites and one cAMP-responsive element [3]. These observations confirmed the critical role of Ad4BP as a steroidogenic cell-specific transcription factor regulating the expression of steroidogenic *P*450 genes.

Ad4BP AND ELP

Ad4BP is a member of steroid hormone/thyroid hormone receptor superfamily, whose members have a zinc finger domain and regions II, and III, which are highly conserved in the superfamily. When the amino acid sequence of Ad4BP was compared with other zinc finger proteins, it showed high homology with ELP and FTZ-F1 (Fig. 2). The zinc finger domain of Ad4BP was 100 and 85% homologous to those of ELP and FTZ-F1, respectively, whereas its homology with the other members of the superfamily was 40–60%. FTZ-F1 is a transcriptional factor of *Drosophila*, which regulates the *fushi tarazu* gene at an early stage of embryonic development. ELP was cloned as a mammalian homolog of FTZ-F1 from undifferentiated murine embryonal carcinoma cells, and suppresses the transcription of Moloney leukemia virus in the cells [8]. We cloned the rat gene of Ad4BP, which consists of 7 exons divided by 6 introns and spans about 15 kb, and confirmed the transcription of Ad4BP and the rat homolog of ELP from the same gene by alternative usage of the promoters and the splice sites (Fig. 3) as recently reported for the mouse homologs of FTZ-F1 [9].

It was found that Ad4BP and ELP recognize the same nucleotide sequences, but, judging from the gel shift assays, the binding of ELP to the Ad4 site was weaker than Ad4BP. The RT-PCR with ELP-specific primers confirmed the expression of ELP in all the steroidogenic tissues of rats where Ad4BP was also expressed, but the expression levels of ELP in the tissues were always significantly lower than those of

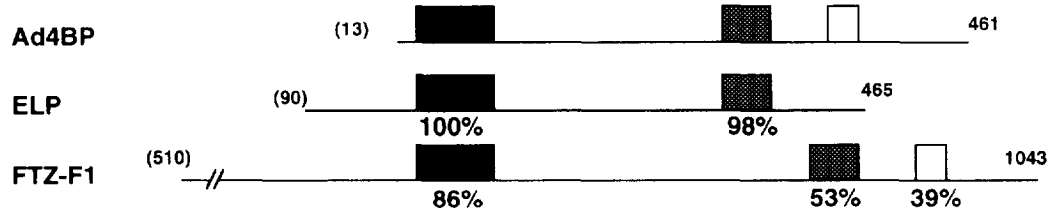

Fig. 2. Schematic comparison of the structures of Ad4BP, ELP, and FTZ-F1 [6]. Regions I, II, and III are shown by the closed, shaded, and open squares, respectively. The number below each square indicates the percentage of similarity in each region as compared with the corresponding region of Ad4BP. The numbers in the parentheses on the left indicate the numbers of the amino acids preceding region I. The numbers on the right indicate the total number of amino acids of each receptor.

Ad4BP [10]. The transcription activity of ELP was examined with the CAT reporter gene under the control of $P450_{11\beta}$ gene promoter. In contrast to the transcription activation by Ad4BP, the cotransfection of an ELP expression vector with the CAT construct into CV-1 cells did not activate the transcription of the CAT gene even in the presence of the PKA expression vector. The CAT activity supported by the expressed Ad4BP was rather suppressed by the coexpression of ELP in the CV-1 cells, although the suppression by a 10-fold excess amount of ELP was only about 50% [10]. We conclude that ELP makes no significant contribution to the tissue-specific expression of steroidogenic *P*450 genes, although ELP coexpresses with Ad4BP in the steroidogenic tissues.

EXPRESSION OF Ad4BP IN STEROIDOGENIC TISSUE CELLS

The expression of Ad4BP mRNA in rat and bovine steroidogenic tissues, adrenal gland, ovary, testis, placenta, adipose tissue, and brain, was confirmed by Northern blotting and RT-PCR [6]. However, these tissues are constituted of several cell types including non-steroidogenic cells. To confirm the steroidogenic cell-specific expression of Ad4BP, we prepared rabbit antiserum to the recombinant Ad4BP expressed in *Escherichia coli* from its full-size cDNA and purified to homogeneity, and used the antiserum for immunohistochemical examination of the steroidogenic tissues of adult rats [10, 11]. Immunoblot analyses of Ad4BP and steroidogenic *P*450s in the corresponding bovine tissues were also carried out.

Adrenal cortex consists of three distinguishable zones, zona glomerulosa, zona fasciculata, and zona reticularis. The glomerulosa and fasciculata/reticularis zones were separately prepared from bovine adrenal gland, and Western blot analyses of Ad4BP and a steroidogenic *P*450, $P450_{scc}$, were carried out. The immunoblot showed almost uniform distribution of Ad4BP, whereas the expression of the *P*450 protein was more abundant in the fasciculata/reticularis than in the glomerulosa. Immunohistochemical staining of the rat adrenal glands showed the expression of Ad4BP in all the cells of the three zones of the cortex at nearly the same extent, whereas the capsule and the medulla were not stained (Fig. 4). Staining by the antibody was exclusively localized to the cell nuclei. The preimmune serum gave no signal.

The ovary contains different types of steroidogenic

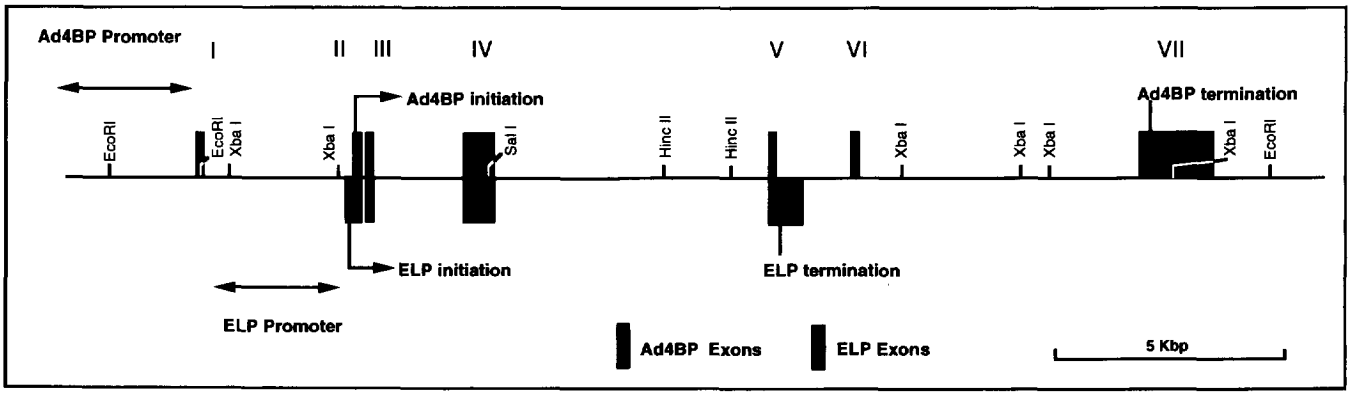

Fig. 3. Structure of rat Ad4BP gene. The closed boxes with numbers indicate the locations and sizes of the exons of Ad4BP gene. The shaded boxes indicate those of ELP. The initiation methionines and the termination codons for Ad4BP and ELP are indicated.

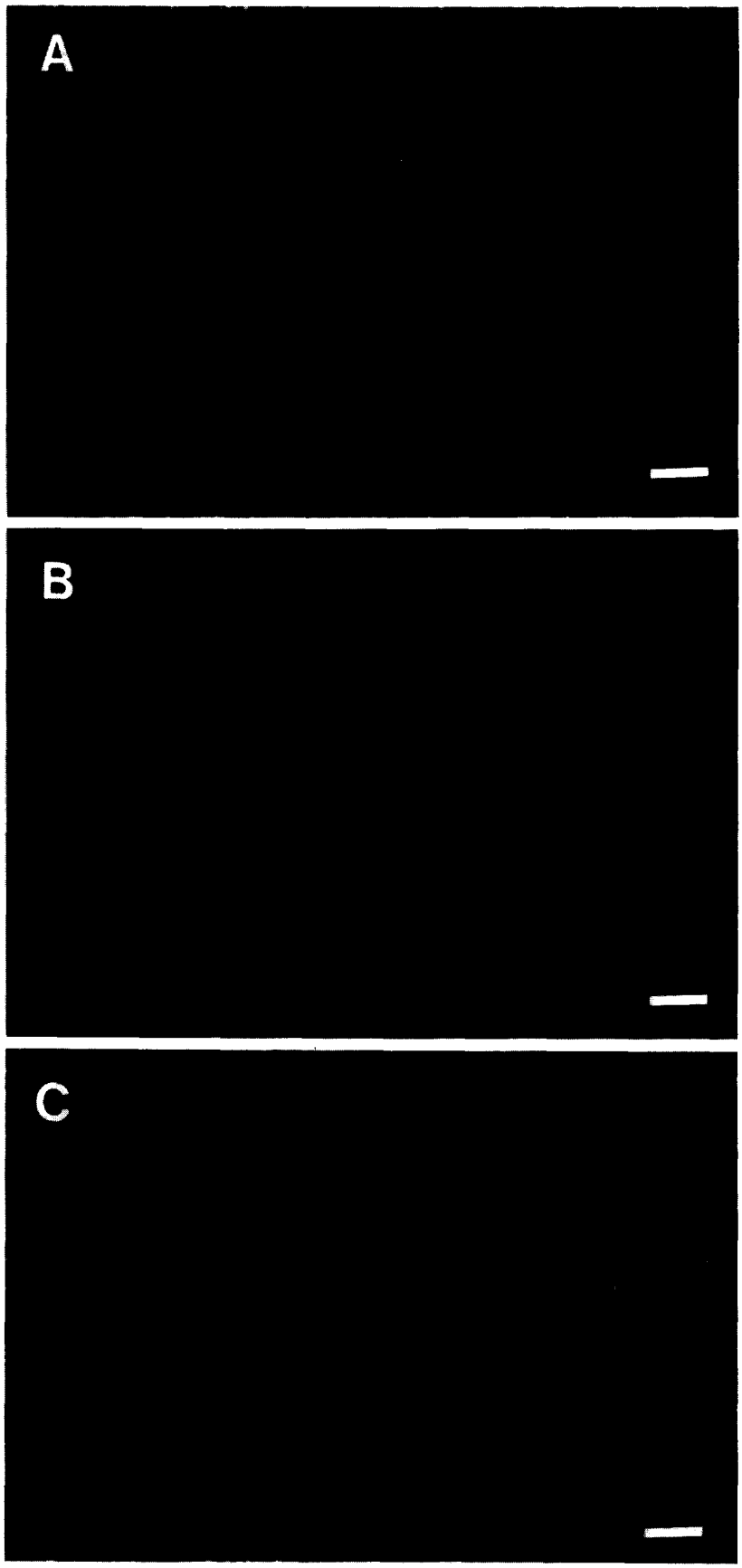

Fig. 4. Localization of Ad4BP in adrenal gland (A), testis (B), and ovary (C) of 8-week-old rats. Frozen sections of the tissues were stained with the antiserum to Ad4BP, followed by FITC-labeled secondary antibody. Bars, 80 μm.

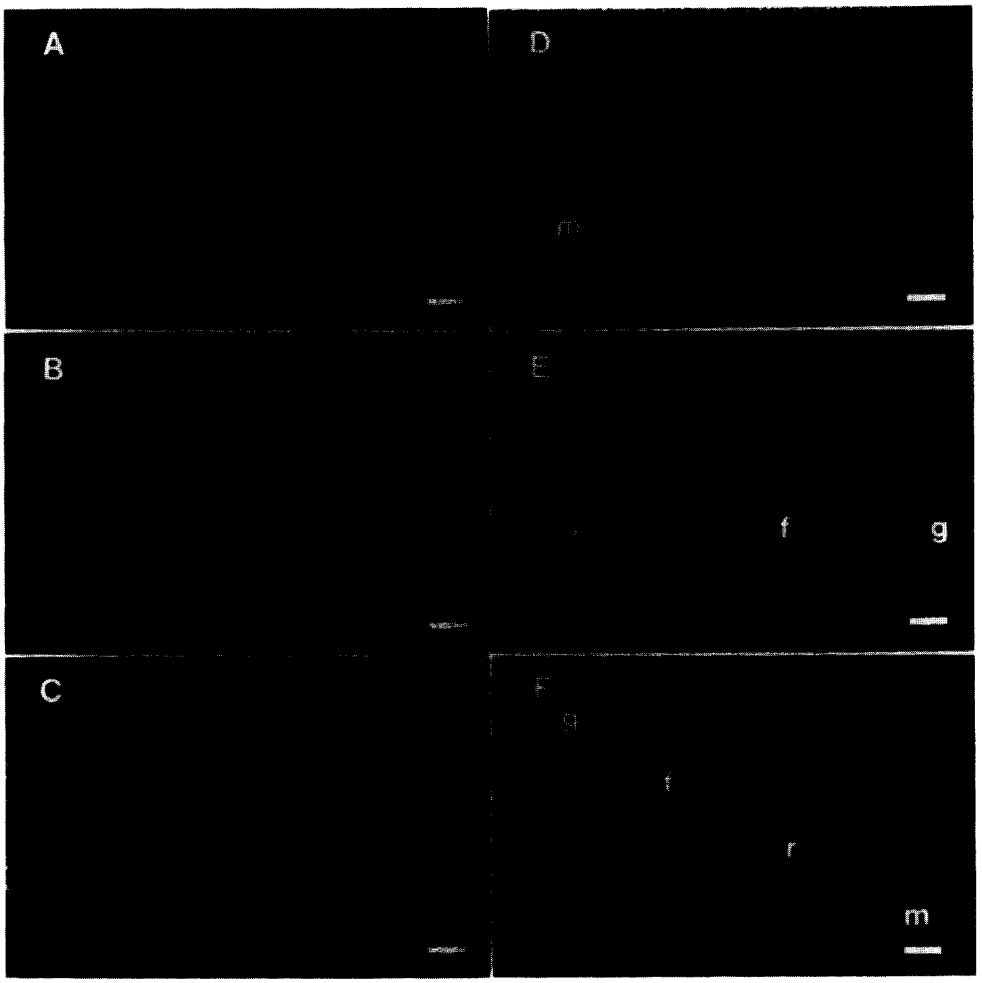

Fig. 5. Expression and localization of Ad4BP in the adrenal glands of fetal and postnatal rats [11]. The adrenal glands shown in A–C were from the male fetuses of 14.5, 16.5 and 20.5 d.p.c., while those shown in D and E were from 2- and 8-week-old male animals, respectively. F shows the adrenal gland of an 8-week-old male fetus stained with the antiserum preincubated with purified Ad4BP. g, zona glomerulosa; f, zona fasciculata; r, zona reticularis; m, medulla. Bars, 80 μm.

cells, the granulosa cells and the theca cells in the follicle, and the corpus luteum cells. The immunoblot analysis of Ad4BP and $P450_{scc}$ with the granulosa cells and the matured and regressed corpus luteum cells prepared from bovine ovaries showed the presence of both proteins in all of the preparations, although the signals of the regressed corpus luteum preparations were significantly weaker than the matured corpus

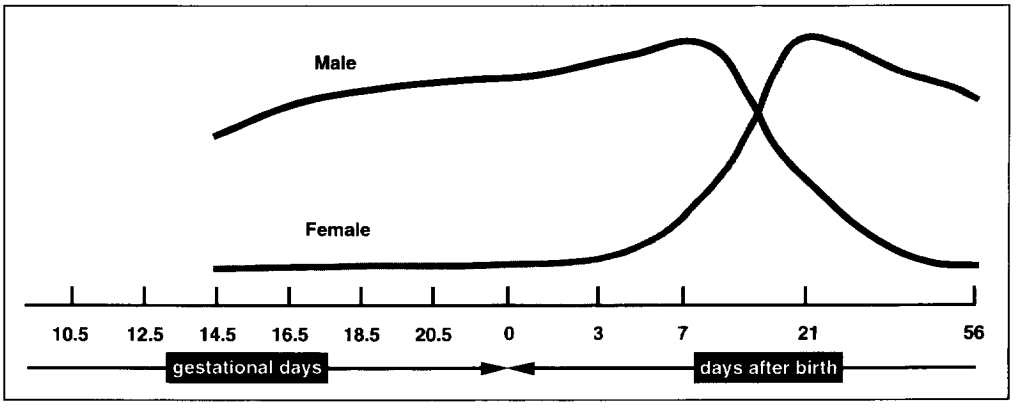

Fig. 6. Time-courses of expression of Ad4BP in the male and female gonads during the development of fetal and postnatal animals. The expression level of Ad4BP in the gonads is indicated on the axis of ordinates.

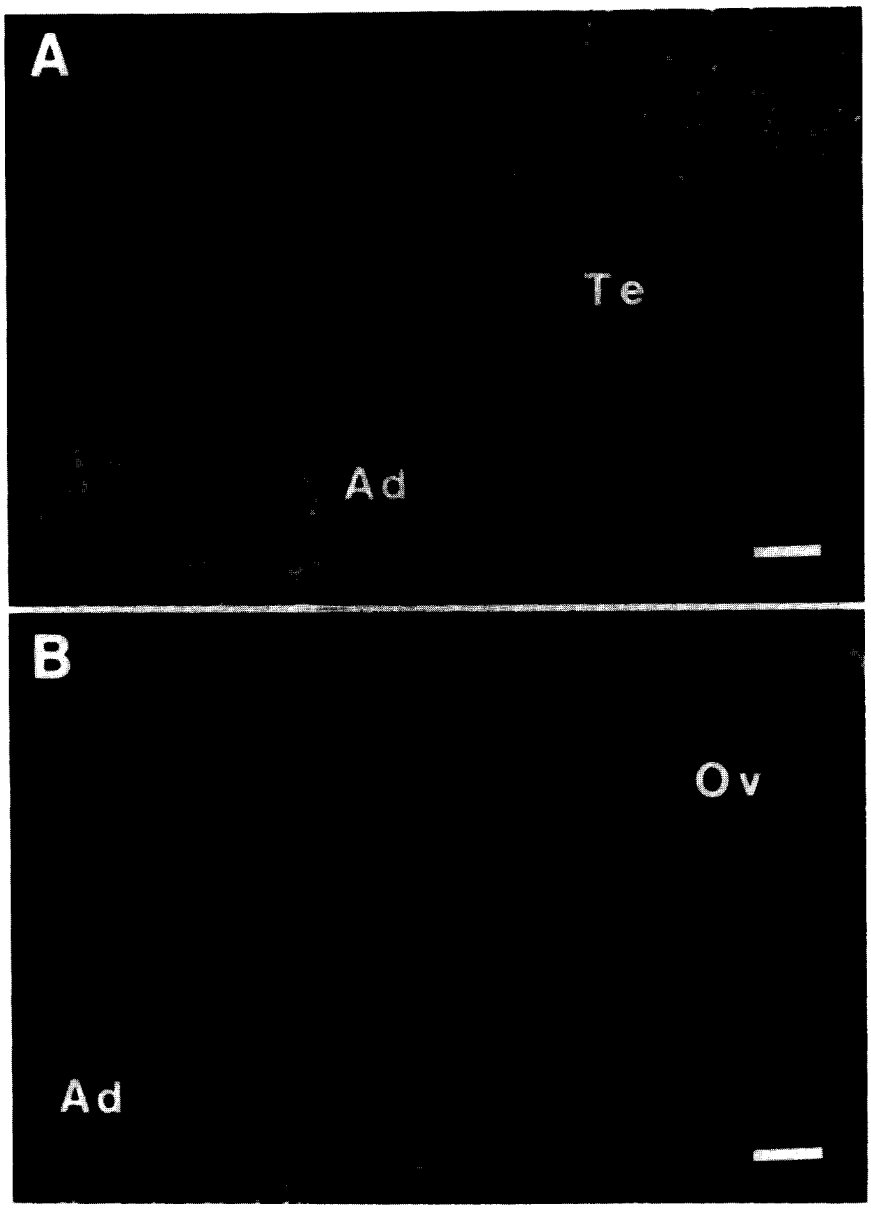

Fig. 7. Comparison of the expression levels of Ad4BP between the male and female gonads using adrenal gland as the control [11]. The sections containing both the gonadal tissue and the adrenal gland were prepared from male (A) and female (B) fetuses of 14.5 d.p.c., and stained with the antiserum to purified Ad4BP. Ad, adrenal gland; Te, testis; Ov, ovary. Bars, 80 μm.

luteum. The regression of the corpus luteum seems to be accompanied by a concomitant decrease in the contents of Ad4BP and steroidogenic *P*450s. Immunohistochemical staining of rat ovaries with Ad4BP antiserum showed intense staining of the theca cells surrounding the follicles, whereas the staining of the granulosa cells in the follicles was significantly weaker (Fig. 4). It is known that $P450_{scc}$ and $P450_{17\alpha}$ are expressed in the theca cells and corpus luteum cells, whereas the granulosa cells contain only $P450_{arom}$. The staining of those cells by anti-Ad4BP antibody coincides with the expression of steroidogenic *P*450s in those cells.

Immunohistochemical staining of rat testis with Ad4BP antiserum showed intense staining of the cells surrounding the seminiferous tubules (Fig. 4). The majority of the cells outside the tubules are Leydig cells, which contain steroidogenic *P*450s and produce androgens. Staining was not detected in the other components of the testis, including the germ cells and Sertoli cells in the seminiferous tubules and the tunica albuginea.

These immunochemical and immunohistochemical observations on the three steroidogenic tissues clearly indicate the spatial coincidence between the expression of steroidogenic *P*450s and that of Ad4BP. Ikeda *et al.* [9] examined the distribution of SF-1 (Ad4BP) transcript in mouse steroidogenic tissues by *in situ* hybridization, and their results agree with ours using Ad4BP antibody.

EXPRESSION OF Ad4BP IN THE ADRENAL GLANDS OF FETAL AND POSTNATAL ANIMALS

To examine the correlation between the expression of Ad4BP and steroidogenic *P*450s, the adrenal glands were obtained from fetal and postnatal rats ranging from 14.5 day postcoitum (d.p.c.) fetuses to 8-week-old animals of both sexes. Immunoblot analysis confirmed the expression of Ad4BP in the adrenal glands of 14.5 d.p.c. fetuses. The expression increased slightly at 16.5 d.p.c., maintained the level until 3 weeks after birth, and then decreased slightly afterwards. On the other hand, the expression of one of the steroidogenic *P*450s, $P450_{scc}$, in the adrenal gland was very low at 14.5 d.p.c., clearly detectable at 16.5 d.p.c., followed by a gradual increase during fetal life. No difference in the expression of Ad4BP and $P450_{scc}$ was detected between male and female animals during the fetal and postnatal periods.

Immunohistochemical staining of Ad4BP confirmed its expression in the fetal adrenal cells at 14.5 d.p.c. On successive days, the cells unstained with Ad4BP antiserum appeared in the medial area, and formed unstained medulla after birth, whereas the stained cortical cells formed three distinct zones during 2–8 weeks after birth (Fig. 5). The intensity of the staining of the adrenocortical cell nuclei by Ad4BP antiserum was almost constant at various developmental stages of animals.

EXPRESSION OF Ad4BP IN THE GONADS OF FETAL AND POSTNATAL RATS

Gonadal sex of rats could be clearly distinguished at 14.5 d.p.c. by the presence or absence of the testicular cord. Western blotting of the male gonads of 14.5 d.p.c. fetuses showed significantly higher expression of Ad4BP than female. The expression of Ad4BP in the testis increased at 16.5 d.p.c., and was almost constant thereafter until birth, while the Ad4BP in the ovary remained low. The testes of neonatal animals kept the high level of Ad4BP expression during the first week, and then a marked decrease occurred at the third week. In contrast, the ovaries became active in Ad4BP expression on the seventh day after birth, and continued to be active until the third week. Figure 6 shows the time-course of the expression of Ad4BP in the testis and the ovary during the development of fetal and postnatal rats.

The expression of $P450_{17\alpha}$, which participates in the synthesis of both androgens and estrogens in the gonadal tissues, was also examined by Western blotting. In the male gonads, $P450_{17\alpha}$ was not detectable at 14.5 d.p.c., but expressed abundantly from 16.5 d.p.c. until birth. The content of $P450_{17\alpha}$ in the testis decreased gradually after birth although Ad4BP continued to express at a high level. On the other hand, $P450_{17\alpha}$ was not detected in the fetal ovaries until birth, and also in the ovaries of neonatal animals. A large amount of $P450_{17\alpha}$ appeared in the ovary of 3-week-old animals. The expression of $P450_{17\alpha}$ in the gonadal tissues of both sexes showed good correlation with that of Ad4BP, which always preceded the former.

Immunohistochemical staining of the male gonads of 14.5 d.p.c. fetal rats showed high expression of Ad4BP in the interstitial cells and the cells surrounding the germ cell aggregates (Fig. 7). The germ cells were not stained. Shortly before birth, two types of Ad4BP-positive cells were detected in the testis. The intensely stained cells seemed to be Leydig cells, whereas the weakly stained cells were Sertoli cells. The staining of the Leydig cells was almost constant after birth, while that of the Sertoli cells decreased afterward.

Ad4BP staining was also detected in the female gonads of 14.5 d.p.c. fetal rats, and the intensity of the staining of the cell nuclei was almost constant during the fetal period when the follicles were not yet formed (Fig. 7). The follicles appeared at 1 week after birth. All the granulosa cells in the preantral follicles were stained, while those in the follicles at earlier stages of development were less intensely stained. Interstitial theca cells were also stained.

POSSIBLE ROLE OF Ad4BP IN SEXUAL DIFFERENTIATION OF GONADAL TISSUES

The expression of Ad4BP in the early gonadal tissues and its sexually dimorphic expression suggests a role of this transcription factor in the sexual differentiation of the gonadal tissues. The sexual differentiation of fetal animals is believed to be triggered by the transient expression of the SRY gene in the somatic cells of the urogenital ridge. The sexually dimorphic expression of Ad4BP observed in the gonads shortly after the sexual differentiation of the fetus suggests that the Ad4BP gene might be one of the genes located just downstream to SRY. A recent paper by Shen *et al.* [12] reported that SF-1 (Ad4BP) regulates the expression of the Müllerian inhibiting substance (MIS) gene, whose product causes the regression of the Müllerian duct in developing male fetus. There is a highly conserved Ad4 site approx. 60 bp upstream from the TATA box in the MIS genes of several animal species [11]. These lines of evidence suggest that Ad4BP regulates the genes essential for the sexual differentiation of the gonadal tissues in fetal animals, in addition to the genes of steroidogenic *P*450s.

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