# Identification of Genes Involved in the Onset of Female Puberty of Rat

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**Onset of female puberty follows a series of prepubertal cellular and molecular events including changes of synaptic plasticity, synthetic and releasing activity and gene expression. Dramatic increase of gonadal steroid level is one of the most prominent changes before the onset of puberty. Based on the importance of steroid feedback upon the hypothalamus, we adopted an estrogen sterilized rat (ESR) model where 100 ng of 17β-estradiol were administered into neonatal pubs for 7 days after birth. To identify genes involved in the onset of female puberty, we applied PCR differential display using RNA samples derived from ESR and control rat hypothalami. About 100 out of more than 1000 RNA species examined displayed differential expression patterns between a 60-day old control rat and ESR. Sequence analysis of differentially amplified PCR products showed homology with genes such as mouse kinesin superfamily-associated protein 3 (KAP3) and several cDNAs previously described by others in mouse and human tissues. Several gene products such as 2-1 and 8-1 corresponded to novel DNA sequences. We analyzed mRNA levels of KAP3, 2-1 and 8-1 genes in the hypothalami derived from neonatal, 6-, 28-, 31-, and 40-day old rats. Northern blot analysis showed that mRNAs of KAP3, 2-1 and 8-1 genes were markedly increased before the initiation of puberty. Neonatal treatment of estrogen clearly inhibited prepubertal increases in KAP3, 2-1 and 8-1 mRNA levels. Therefore, these genes may play important roles in the initiation of hypothalamic puberty. In addition, intracerebroventricular (icv) injection of antisense KAP3 oligodeoxynucleotide (ODN) clearly delayed puberty initiation determined by vaginal opening, which further confirmed that KAP3 plays an important role in the regulation of puberty initiation.**

Sexual maturation of the female hypothalamus was known to begin with a process of functional and structural differentiation in response to gonadal steroids during a 'critical period' of neuronal development (reviewed by Gorski, 1985) and be completed in the onset of puberty (reviewed by Ojeda and Urbanski, 1988). It was previously demonstrated that the onset of puberty in the female rat is characterized by a gradual increase in circulating sex steroid levels and the occurrence of the first preovulatory surge of gonadotropins (Ojeda et al., 1976; Parker and Mahesh, 1976).

In the rodent, the 'critical period' of brain sexual differentiation is usually from late pregnancy to 7 to 10 days after birth (vom Saal and Bronson, 1980; Weisz and Ward, 1980). During the 'critical period', estrogen exerts organizational effects on the differentiation of a number of sexually dimorphic brain areas and permanently modulates sex-related brain activities and behaviors to be masculinized in adult mammals (Parsons et al., 1980; Toran-Allerand, 1984; McEwen et al., 1987). In the normal female rat during the late gestational and early postnatal ages, the brain is protected by fetoneonatal estrogen binding protein (FEBP) from being organized as male-like characters (Gorski 1985). In contrast, circulating testosterone in the male is free from binding to FEBP and can reach the brain. In the brain, testosterone is converted to estradiol by the action of aromatase and organizes specific characters in the brain of males, including masculine behaviors, tonic gonadotropin release mechanisms, and various morphological indices (Gerall et al., 1967; Raisman and Field, 1973; Greenough et al., 1977; Barraclough, 1979; Matsumoto and Arai, 1981).

Even in the female, excess amounts of estrogen can overcome the saturation of FEBP and act on the brain to be masculinized as described below. The treatment of neonatal rats with a high dose of estrogen (or androgen) resulted in permanent sterility during adulthood (Barraclough, 1961; Gorski, 1963; Hayashi and Aihara, 1989; Hayashi et al., 1991). The female estrogen sterilized rat (ESR) showed masculinized characters

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and an enlarged sexually dimorphic nucleus in the preoptic area (SDN-POA) compared to normal females (Faber and Hughes, 1991). Puberty was clearly delayed and the gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) surges disappeared by destruction of hypothalamo-pituitary-gonadal axis (Hayashi and Aihara, 1989; Faber et al., 1991; 1993; Pinilla et al., 1993). The sterility observed in the ESR was reported to be due to the lack of neural circuits regulating the release of GnRH (Borvendeg et al., 1972; Mennin et al., 1974; Hayashi et al., 1991). Together with these results, neonatal treatment of estrogen may cause changes in the organizational pattern of the brain areas responsible for the sexual differentiation and behaviors preceding the normal pubertal process. Studies thus far are not sufficient for us to understand the molecular mechanisms underlying irreversible sexual differentiation in the brain imprinted by the organizational effect of gonadal steroids.

Estrogen regulates gene transcription by binding with its receptor to specific regulatory DNA sequences within the promoter of target genes (Evans, 1988; Kumar and Chambon, 1988; Klein-Hitpass et al., 1989). Biochemical studies reported that the presence of estrogen receptors in developing rodent brains coincides with the 'critical period' of responsiveness to the organizing effects of estrogen (Gerlach et al., 1983; Sibug et al., 1991). Several studies in neural cells or tissues reported that estrogen regulates transcription for specific proteins, including synapse associated proteins (Shughrue and Dorsa, 1993), neurotrophins (Toran-Allerand, 1996), neurotransmitter receptors (Summer and Fink, 1993), protooncogenes (Pahlman et al., 1990; Santagati et al., 1995), and growth-associated nuclear protein prothymosin-α and Bcl2-interacting protein Nip2 (Garnier et al., 1997).

Using the ESR animal model and differential display PCR (ddPCR) technique (Liang and Pardee, 1992; Douglass et al., 1995), we aimed to identify genes involved in the onset of puberty. In this study, we identified several genes which may explain the processes of hypothalamic sexual differentiation and the onset of puberty at the molecular level.

## Materials and Methods

### *Animals, tissue and RNA preparation*

Pregnant female rats of the Sprague Dawley strain were maintained *ad libitum* in a condition of 14-h light and 10-h dark photocycle. Neonatal pubs were kept with their mother and daily sc injected with 100 ng of 17β-estradiol for 7 days. 17β-estradiol was dissolved in ethanol and further diluted with sesame oil. Control animals were daily sc treated with sesame oil containing the same amount of ethanol for 7 days. The females of estrogen sterilized rats (ESRs) and control animals in random phases of the estrous cycle were sacrificed at 60 days of age and total hypothalamic tissues including the preoptic area (POA) and medial basal hypothalamus (MBH) were removed and pooled from 6-10 animals. When animals were sacrificed, ovaries and uteri were collected and weighed to determine the effect of neonatal treatment of estrogen. All animals that received estrogen neonatally showed a clear regression of reproductive organs such as the ovary and uterus (data not shown).

RNA extraction from hypothalamic tissues of ESR and control animals was generally performed with an acid phenol-chloroform method (Chomczynski and Sacchi, 1987). Total RNA samples were first digested with 10 U of DNase I for 30 min at 37℃ to remove possible DNA contaminants. After phenol-chloroform extraction and ethanol precipitation, RNA was diluted to a concentration of 0.1 μg/μl in RNase free water.

### *Differential display PCR (ddPCR)*

RNA samples (2 μg) from ESR and control animals were reverse transcribed using 50 pmole of anchor oligo-dT primer ($dT_{12}VA$, $dT_{12}VC$ and $dT_{12}VG$, Operon) and 200 U of MMLV reverse transcriptase (Promega) to generate different cDNA fractions. Reverse transcribed cDNA was applied to PCR amplification using anchor oligo-dT primer and random 10-mer primer sets (Operon) as 3' and 5' primers, respectively. We added 4 μCi of $^{35}$S-dATP to the reaction mixture to analyze the PCR product by autoradiography. Forty cycles of PCR were performed by condition of 1 min at 95℃ denature, 1 min at 45℃ for annealing and 1 min at 72℃ for extension.

Radiolabeled PCR products were separated and analyzed by a high resolution sequencing gel containing 6% polyacrylamide. Dried gel was autoradiographed for 3 days onto X-ray film. If the results of autoradiogram showed density differences between PCR products from ESR and control animals, we excised the bands with razor blades and extracted PCR products from the gels. Extracted ddPCR products were amplified again under the same PCR conditions without isotopes. We checked the sizes of reamplified PCR products in a 2% agarose gel.

### *Reverse northern blot hybridization to validate ddPCR products*

Reverse northern blot hybridization analysis was performed to confirm that the amplified PCR products were not artificial. Reamplified PCR products were blotted onto a positively charged $N^+$ nylon membrane (Amersham) in duplicates and hybridized with cDNA probes which were synthesized by reverse transcription from RNA extracted from ESR and control animal hypothalami in the presence of $^{32}$P-dCTP. One part of duplicate membrane sets was hybridized with cDNA from ESR animals and the other part was hybridized with cDNA from control animals. After hybridization, membranes were washed 3 times with washing solution

containing 2x SSC (1x SSC: 0.15 M NaCl and 0.015 M Na-citrate) and 0.1% SDS for 15 min at 42℃ and followed by a second washing step with 0.5% 2x SSC solution and 0.1% SDS for 15 min at 42℃. Membranes were dried and autoradiographed onto the X-ray film for 24 h.

*Cloning, sequencing and analysis of PCR products*

PCR products of which blots showed clear density difference in the reverse northern blot hybridization method were chosen, cloned into the pGEM-T easy vectors (Promega), and sequenced with an automatic DNA sequencer (Korea Basic Science Institute). The DNA sequence of PCR products were compared with GenBank at the National Center for Biotechnology Information (NCBI), using the BLAST algorithm on the "nr" nonredundant database, and described in Table 1. In many cases, sequences gave no match to the rat sequences but close matches to mice or other known organisms. These sequences were assumed to be the rat homologs of known genes. Sequences that gave no matches with the known genes were further compared with the GenBank "dbest" database. Many sequences were found to have close matches to the mouse and human expressed sequence tags. Novel genes described in Table 1 did not show any sequence homology with nr and dbest of the GenBank database.

*PCR cloning of rat homolog with mouse KAP3 cDNA*

In case of the rat homolog with mouse KAP3 cDNA, the partial cDNA fragment was cloned by PCR using primer sets based on the known sequence (Yamazaki et al., 1996). This PCR-cloned partial cDNA fragment was used as a template for the generation of the cRNA probe for *in situ* hybridization histochemistry. For PCR amplification of KAP3 cDNA, the upstream primer (5′-TGT TGG TGA AGG CTC TTG ATC G -3′) and down stream primer (5′-TGC AGT GAG CTT TGG GAG AAG C -3′) were designed. Amplification products were inserted into pGEM-T easy vector (Promega) and sequenced using an automatic DNA sequencer.

*Phases of postnatal development*

Changes in hypothalamic mRNAs of ddPCR-cloned genes were determined at 0-, 6-, 28-, 31- and 40-day old in ESR and control animals. Animals studied for the determination of KAP3 mRNA level during the initiation of puberty were classified according to the previous report (Ojeda and Urbanski, 1988). Briefly, rats were considered to be in the anestrous phase of puberty if their vagina had no sign of activation and a uterine weight of 60 mg or less, without accumulation of intrauterine fluid. Animals with enlarged uteri and detectable intrauterine fluid were classified in the early proestrous stage, which precedes the day of the first preovulatory surge of gonadotropins. Animals with a uterine weight of at least 200 mg and a uterus ballooned with fluid were considered to be in late proestrus, that is the phase of puberty during which GnRH and gonadotropins are for the first time discharged as a preovulatory surge. The first ovulation occurs on the next day (first estrus). At this time, the vagina becomes patent exhibiting a predominance of cornified cells, and ovaries have fresh corpora lutea.

*Northern blot hybridization*

Northern blot hybridization was carried out to determine changes in hypothalamic mRNAs of ddPCR products. Total hypothalamic RNA was extracted and pooled from more than 10 animals at the same ages and aliquots of RNA (20 µg) were separated on a 1.2% formaldehyde-agarose gel, and transferred onto a nylon membrane. Membranes were hybridized with radio-

**Table 1.** Identification of ddPCR-cloned genes and density differences between hybridization signals with 2 probes in reverse northern blot analysis

| Clone | Homology analysis | Species | Density differences in reverse northern blot analysis | |
|---|---|---|---|---|
| | | | Control probe | ESR probe |
| 1-1A | testis specific X-linked gene | mouse | + | ++ |
| 1-3 | novel | | +++ | + |
| 1-5 | tryptophan hydoxylase gene | mouse | + | ++ |
| 2-1 | novel | | - | +++ |
| 2-2 | novel | | ++ | +++ |
| 2-3 | novel | | + | - |
| 2-4 | surfeit-6 gene | mouse | ++ | - |
| 5-1A | pooled organ cDNA | mouse | - | ++ |
| 5-2 | novel | | + | ++ |
| 5-3 | novel | | - | + |
| 8B | kinesin superfamily associated protein (KAP3) | mouse | + | +++ |
| 8-1 | novel | | + | +++ |
| 12-1 | novel | | +++ | ++ |
| 13-2 | pooled organ cDNA | mouse | ++ | + |
| 14-2 | novel | | + | - |
| 15-2 | novel | | +++ | - |
| 16-1 | apolipoprotein B editing enzyme gene | mouse | + | - |
| 17-1 | Ser Thr kinase gene | mouse | ++ | +++ |
| 18-3 | novel | | ++ | +++ |
| 24-1 | novel | | +++ | ++ |
| 24-2 | cDNA | human | + | ++ |
| 25-1 | pooled organ cDNA | mouse | - | ++ |

-, no signal; +, week signal; ++, strong signal; +++, very strong signal

labeled probes in hybridization solution at a specific activity of $1 \times 10^6$ cpm/ml. Final posthybridization washes were performed in $0.1 \times$ SSC at 60℃. Autoradiographic images of X-ray film were normalized with densities of 18 S ribosomal RNA bands and calculated as a percent (%) of control values. RNA samples (20 µg) from several tissues including the cerebral cortex, cerebellum, and other peripheral tissues were hybridized with the same cDNA probes, to determine the tissue distribution of the transcripts.

*Probes*

Probes for northern blot hybridization were labeled with $^{32}$P-dCTP (Amersham) using a random primer labeling kit with *Eco*RI digested ddPCR cloned genes. $^{32}$P-labeled probes were separated from the free isotope with a Nick column (Pharmacia). Antisense RNA probe for *in situ* hybridization of KAP3 mRNA was transcribed from the linearized template with RNA polymerase in the presence of $^{35}$S-UTP (Amersham), and separated with a Nick column.

*In situ hybridization histochemistry*

The procedure employed is that described previously (Simmons et al., 1989) with minor modifications. The brains were fixed by transcardiac perfusion with 4% paraformaldehyde in phosphate buffered saline (pH 7.4), and followed by overnight post fixation in the same fixative containing 10% sucrose. After blocking the regions of interest, the tissues were frozen on dry ice and stored at -80℃ until sectioning. Twenty micrometer sections were obtained with a sliding microtome, adhered to ProbeOn™ Plus microscope slides (Fisher Scientific), and dried under vacuum overnight before hybridization. The sections were overlaid with 70 µl of hybridization solution containing $5 \times 10^6$ cpm of probe per ml and hybridized overnight at 60℃. Post hybridization washes were carried out as recommended (Simmons et al., 1989). Some sections were dipped in $NTB_2$ emulsion (Eastman Kodak) and developed after 3 weeks exposure.

*Targeted disruption of KAP3 synthesis*

To examine the involvement of KAP3 in the puberty initiation of female rats, antisense oligodeoxynucleotide (ODN) was delivered to the lateral ventricle via a stereotaxically implanted cannula. Antisense ODN (5′-CTC GCC TTG CAT GGC GGC GGC-3′) was designed to have an antisense sequence against 21 bases including the translation initiation site based on previous data (Yamazaki et al., 1996). ODN, containing the same nucleotide composition of antisense ODN, but in scrambled order (SCR: 5′-TCC GTG GGT CCC GGA GCG CCT-3′), was used as a control. This scrambled sequence does not bear similarity with any sequence present thus far in NCBI GenBank.

For the icv injection, the ODNs were diluted in saline at a final concentration of 0.5 mM. Under pentobarbital (7.5 mg/kg BW) and ketamine hydrochloride (25 mg/kg BW) anesthesia, a polyethylene guide cannula (od 1.05 mm, id 0.35 mm) with an inner stylet (27 gauge) was stereotaxically implanted into a lateral ventricle (1.1 mm rostral to the bregma, 1.7 mm lateral from the midline, and 4.2 mm vertical from the surface of the skull) of 24-day old female SD rats and fixed in place with anchor screws and dental cement. Upon the end of a recovery period of 2 days, the inner stylet was removed and the ODNs (100 ng/rat, respectively) were icv injected with a Hamilton syringe once a day for 2 days. Two days later, after the second delivery of ODNs, we verified the selective effect of antisense ODN on the KAP3 mRNA level using northern blot hybridization analysis. The functional consequence of ODNs on the onset of female puberty was determined by observation of ages at the vaginal opening.

*Statistical analysis*

The effects of ODNs were analyzed with a one-way analysis of variance followed by the Student Neuman-Keul's multiple comparison test for unequal replications.

## Results

*Differential display PCR and reverse northern blot hybridization*

In this study, we attempted to identify genes involved in the pubertal process. As shown in Fig. 1, we

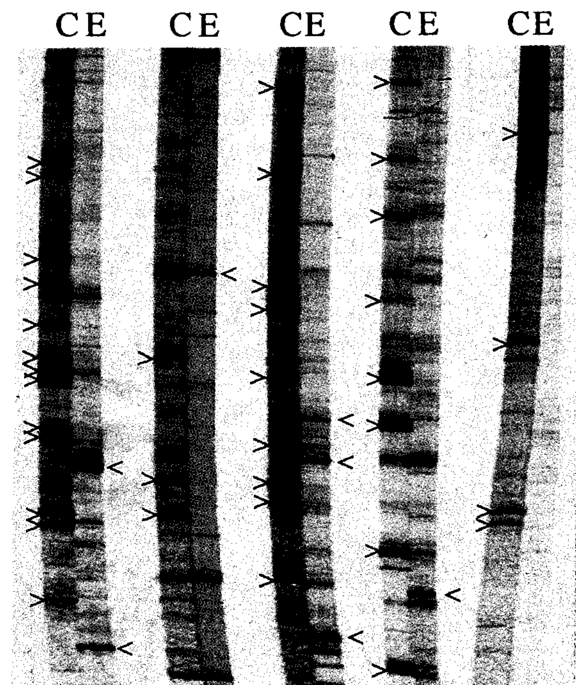


**Fig. 1.** Differential display of mRNAs derived from control (C) and estrogen sterilized rat (E) hypothalami. Bands marked with arrowhead indicate the differential display and were extracted from the gel. The extracted PCR products were reamplified for further study.

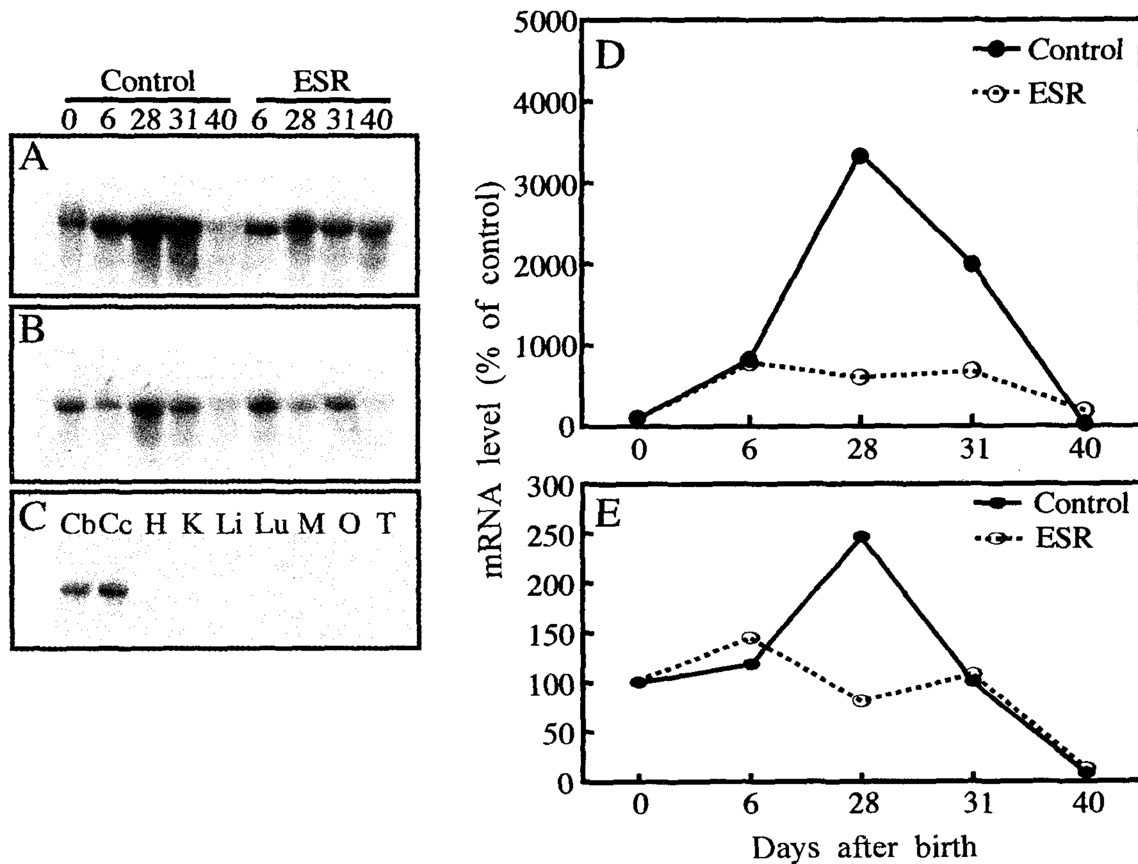


Fig. 2. Northern blot analysis of 8B (KAP3) mRNA (3.5 kb). Radiolabeled cDNA probe was hybridized with hypothalamic RNA (20 μg) derived from 0-, 6-, 28-, 31-, and 40-day old female (A) and male (B) rats. 8B mRNA was found in the cerebral cortex and cerebellum (C). Calculated levels of mRNA increased after birth and reached peak level at the prepubertal 28-day of age in female (D) and male (E) rats. Neonatal treatment of estrogen (ESR) clearly suppressed the prepubertal peaks of mRNA levels observed in the control. Densities of northern blot bands were normalized with 18S ribosomal RNA (data not shown) levels and calculated as % of control (0-day values). Cb, cerebellum; Cc, cerebral cortex; H, heart; K, kidney; Li, liver; Lu, lung; M, muscle; O, ovary; T, thymus.

compared hypothalamic RNAs from ESR with those from control animals using ddPCR. The ddPCR products generated from each reaction were compared side by side after electrophoresis on urea-acrylamide sequencing gels. Neonatal treatment of estrogen induced up-regulation or down-regulation of more than 100 gene transcriptions. Therefore, these could be estrogen-responsive genes involved in the organizational effect of brain development. To confirm that the expressions of these genes in the adult are indeed regulated by the organizational role of neonatal treatment of estrogen, gene products showing differential display were excised out from the gel, reamplified using corresponding primers, and tested further with reverse northern blot hybridization.

*Identification of estrogen-induced genes*

When compared with reverse northern blot hybridization, more than 20 genes showed density differences between the two hybridization probes (Table 1). Genes showing clear density differences in the hybridization between the two probes were cloned into pGEM-T easy vectors, sequenced with an automatic sequencer and analyzed for comparison with the GenBank database (see the Material and Methods for detail). The identification of reamplified PCR products are presented in Table 1. The search for possible homologies, using GenBank and EMBL databases, did not score a high degree of homology for 12 of the ddPCR cloned genes selected, suggesting that they may not be related to any known genes. On the contrary, a high degree of homology (80-100%) was found between 10 ddPCR products and previously reported sequences, such as the mouse testis specific X-linked gene, the mouse tryptophan hydroxylase gene, the mouse surfeite-6 gene, mouse KAP3 cDNA, mouse apolipoprotein B editing enzyme cDNA, and the mouse Ser Thr kinase gene.

*Developmental patterns of gene expression*

We analyzed mRNA levels of ddPCR products that were differentially amplified by neonatal treatment of estrogen using northern blot hybridization. Radiolabeled probes were generated by labeling the *Eco*RI digested

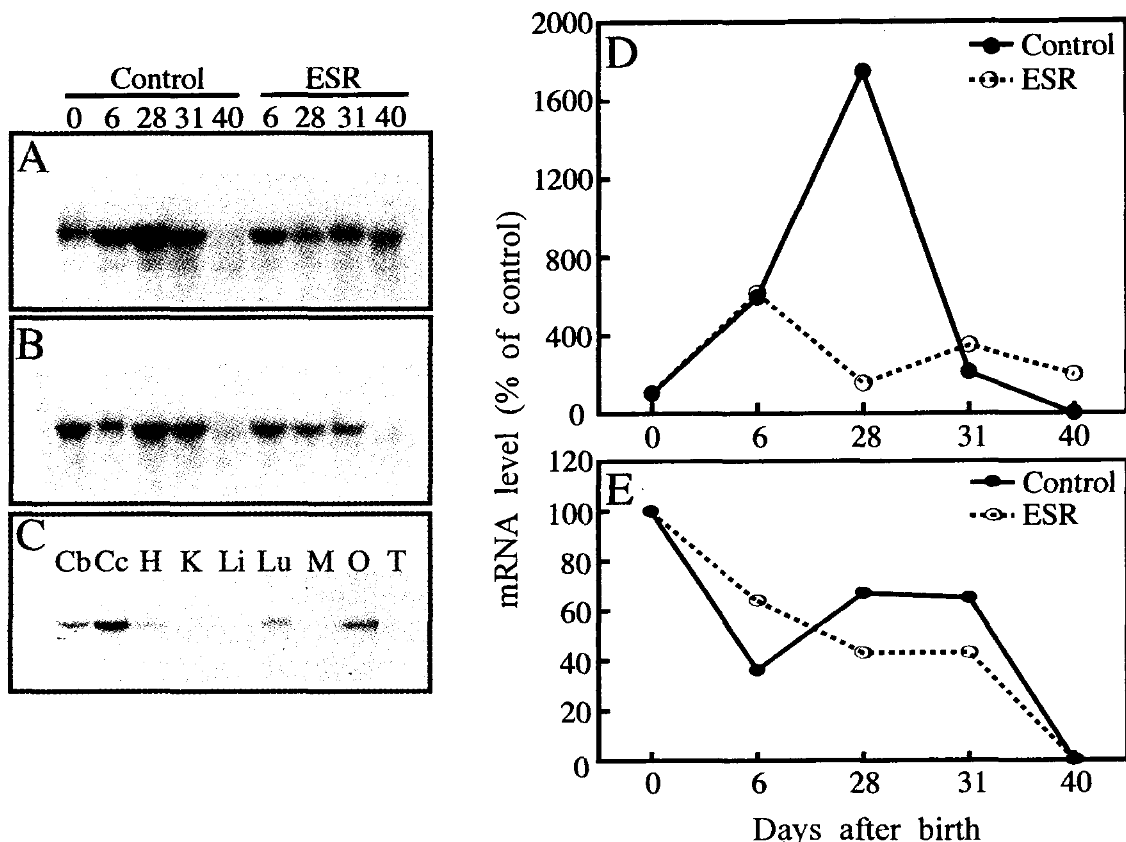


Fig. 3. Northern blot analysis of 2-1 mRNA (4.2 kb). Hypothalamic RNA samples from female (A) and male (B) were hybridized with $^{32}$P-labeled 2-1 cDNA probe. Calculated densities were presented as % of control (0-day values) in panels D (female) and E (male), respectively. In the female, 2-1 mRNA level peaked at the 28-day of age, but neonatal treatment of estrogen (ESR) clearly inhibited the peak level. 2-1 mRNA was found in several tissues including brain regions (C). Abbreviations shown here are the same with those in Fig. 2.

inserts of pGEM-T easy vectors and were hybridized to RNAs from 0-, 6-, 28-, 31- and 40-day old ESR and control rat hypothalami. The hybridization bands were quantitated by densitometric scanning of the autoradiographs, followed by normalization with respect to densities of 18 S rRNA bands stained with ethidium bromide and expressed as percent (%) of 0-day control values.

The 8B probe hybridized with a single transcript of about 3.5 kb (Fig. 2), which corresponds to the reported size of KAP3 mRNA (Yamazaki et al., 1996). Northern blot analysis showed that KAP3 mRNA significantly increased to the peak level at 28-day of age and decreased after the peak in both the female and male rat hypothalamus. On the contrary, neonatal treatment of estrogen removed the 28-day peak of KAP3 mRNA level. KAP3 showed tissue specific expression in the brain regions only among tissues which we selected to determine the transcription (See also the localization of KAP3 mRNA in the brain region, Fig. 5).

Message levels of 2 novel genes (referred to as 2-1 and 8-1) were measured with northern blot analysis. Northern blot hybridization with 2-1 cDNA probe showed a single transcript of approximately 4.2 kb in size in the hypothalamus and other tissues (Fig. 3). Calculation of densities revealed that 2-1 mRNA markedly increased at 28-day of age in the hypothalamus of the female rat, while no such increase was observed in the male rat hypothalamus. Neonatal treatment of estrogen (ESR) clearly suppressed the 28-day peak of the 2-1 mRNA level in the female rat hypothalamus. The same single transcript was found in the brain regions such as the cerebellum and cerebral cortex, and in peripheral tissues such as the lung and ovary.

Hybridization with 8-1 cDNA probe revealed a single transcript of about 3.7 kb in size (Fig. 4). The level of 8-1 mRNA also showed a great increase at the prepubertal 28-day of age in the female rat hypothalamus. Neonatal treatment of estrogen (ESR) inhibited prepubertal increase of 8-1 mRNA in the female rat. Such a dramatic increase at the 28-day of age was not observed in the male rat hypothalamus. A single transcript of the 8-1 gene was found in nearly all tissues determined, such as the cerebellum, heart, kidney, lung, muscle, ovary and thymus.

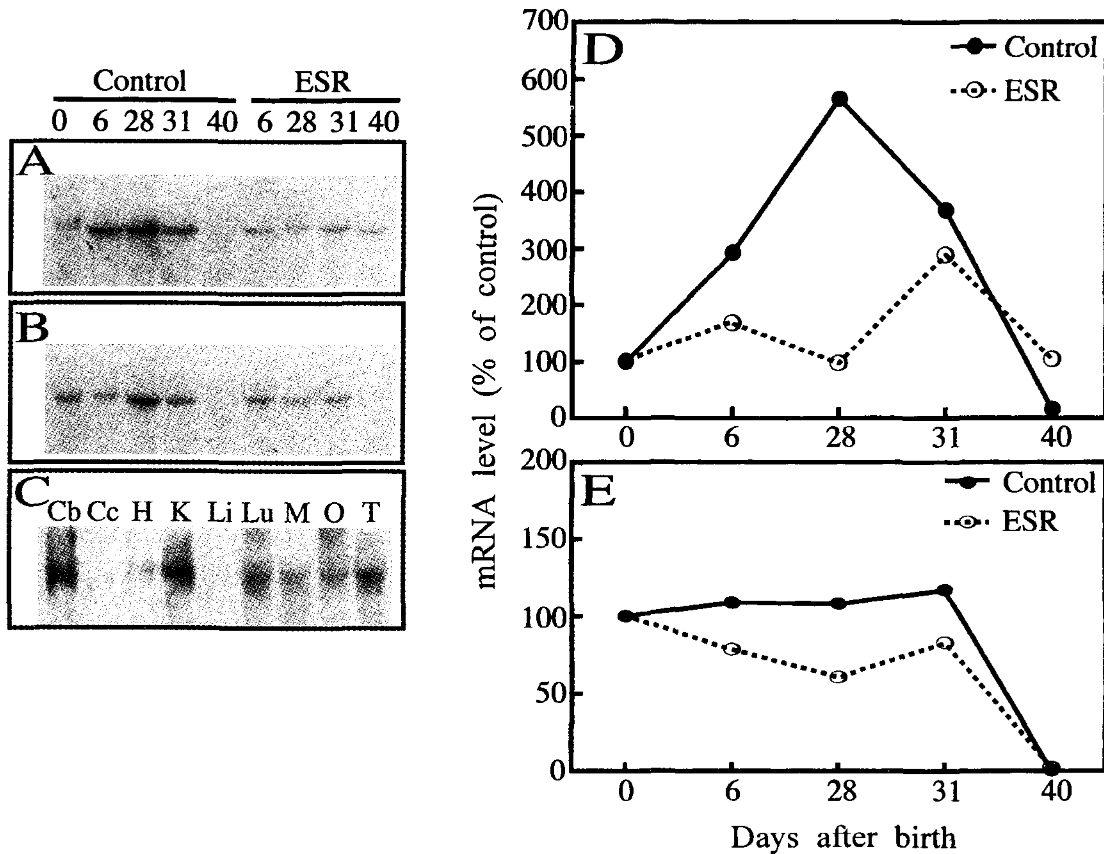


Fig. 4. Northern blot analysis of 8-1 mRNA (3.7 kb). RNA samples were extracted from the hypothalami of 0-, 6-, 28, 31-, and 40-day old female (A) and male (B) rats and hybridized with $^{32}$P-labeled 8-1 cDNA probe. Calculated densities were presented as % of control (0-day values) in panels D (female) and E (male), respectively. A single transcript was found in nearly all tissues determined (C). Abbreviations shown here are the same with those in Fig. 2.

*Localization of KAP3 mRNA*

An antisense RNA probe complementary to the coding region of KAP3 mRNA was synthesized from the PCR cloned cDNA fragment and used to determine the sites of KAP3 mRNA expression in the coronal sections of the 28-day old female rat brain. Although specific labeling was detected in cells scattered throughout the brain, KAP3 mRNA was more abundant in discrete brain areas including the hippocampus, cerebral cortex, piriform cortex and hypothalamic area (Fig. 5). No detectable positive signals were observed in the sections hybridized with a sense probe.

KAP3 mRNA was highly concentrated in the hippocampal layers of CA1, CA2 and CA3, and piriform cortex. KAP3 mRNA was also abundant in the hypothalamic areas including the ventromedial (VMH) and arcuate nuclei (ARC). The ARC and VMH are located in the medial region of the hypothalamus, lateral to the third ventricle in a ventral and intermediate position, respectively, along the sides of the third ventricle.

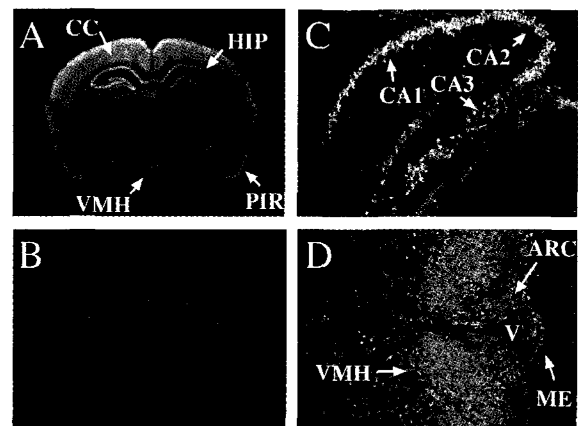


Fig. 5. Localization of KAP3 mRNA in the coronal sections of the female rat brain. Sections were hybridized with $^{35}$S-labeled antisense (A, C and D) or sense (B) probe. Dark field of film autoradiography (A, magnification of 10x) represents the positive signals of KAP3 mRNA in the hypothalamic area including ventromedial hypothalamic nucleus (VMH). Signals were also found in the cerebral cortex (CC), hippocampus (HIP) and piriform cortex (PIR). No such specific signals were found in the section hybridized with a sense probe (B). Dark fields of emulsion autoradiographies (magnification of 40x) show dense signals concentrated in the hippocampal layers of CA1, CA2 and CA3 (C) and medial hypothalamic area such as the median eminence (ME), arcuate nucleus (ARC) and VMH (D). V, third ventricle.

*Prepubertal changes in mRNA level of KAP3*

To determine the changes in the KAP3 mRNA level

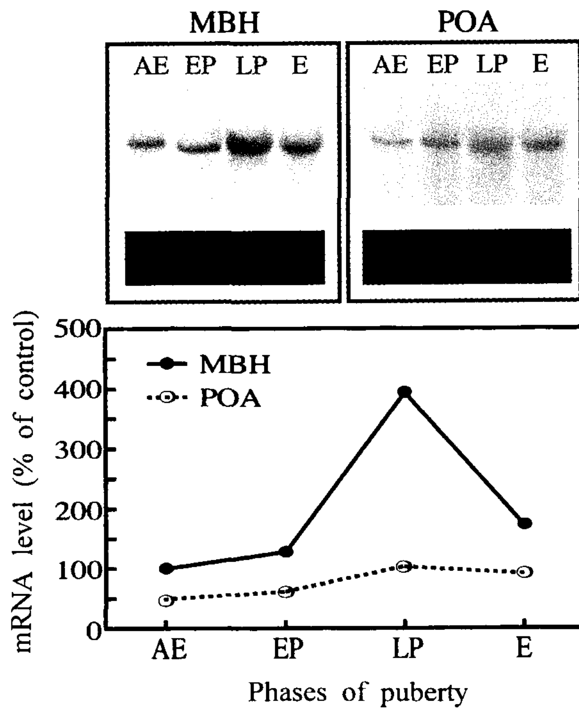


**Fig. 6.** Changes in KAP3 mRNA levels during the pubertal process in the female rat hypothalamus. RNA samples (20 µg) from medial basal hypothalamus (MBH) and preoptic area (POA) were hybridized with a $^{32}$P-labeled cDNA probe. KAP3 mRNA level peaked at late proestrus (LP) in MBH, while no such dramatic change in the KAP3 mRNA level was observed in the preoptic area (POA). Autoradiographic densities were normalized with the densities of 18S ribosomal RNA bands (lower inlets of upper panels), calculated as % of the control (level in AE of MBH) value and presented in the lower panel. Abbreviations: AE, anestrus; EP, early proestrus; LP, late proestrus; E, estrus.

during the prepubertal period, northern blot analysis was carried out with hypothalamic RNA samples from female rats showing anestrus (AE), early proestrus (EP), late proestrus (LP), and first estrus (E). KAP3 mRNA in the MBH tissue showed a peak at LP, while such a peak was not observed in POA (Fig. 6). The prepubertal change in the KAP3 mRNA level in MBH suggested the possible role of KAP3 in the process leading to the onset of female puberty.

*Effect of antisense KAP3 ODN on the onset of puberty*

To block synthesis of KAP3, we introduced antisense KAP3 ODN into the lateral ventricle of the immature female rat. Two days later, we determined mRNA levels of KAP3 to validate the selective effect of ODN. Antisense ODN clearly reduced the level of KAP3 mRNA (upper panel of Fig. 7), which showed the specific action of ODN on KAP3 synthesis. The administration of ODN resulted in a marked delay of puberty, as determined by the age of vaginal opening compared to the sham operated and SCR controls (lower panel of Fig. 7). The functional consequence of antisense ODN showing the delay of vaginal opening further

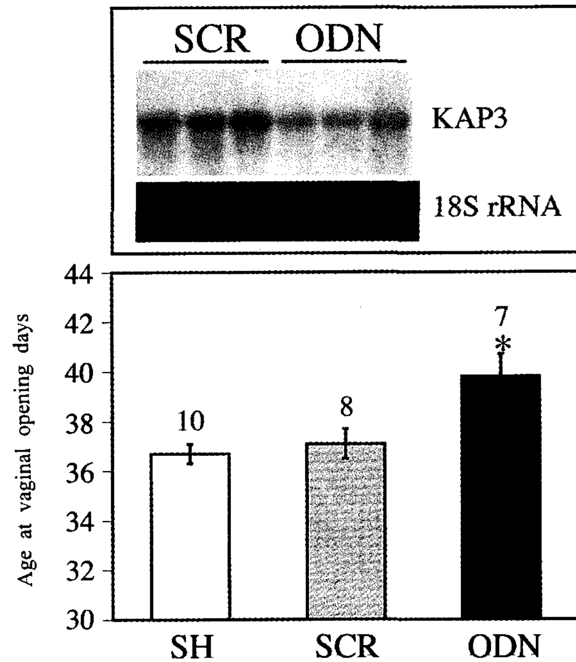


**Fig. 7.** Effect of targeted disruption of KAP3 on the onset of female puberty. Upper panel depicts the specific effect of antisense KAP3 ODN on the KAP3 mRNA level. Antisense ODN clearly decreased the KAP3 mRNA level in comparison with SCR. In the lower pannel, each bar represents the age at vaginal opening in days (x±SEM). The numbers on the top of each bar indicate the number of animals per group. Asterisk indicates $P<0.05$ versus sham operated (SH) or scrambled ODN (SCR) injected control groups.

suggested that KAP3 play an important role in the regulation of the onset of puberty.

## Discussion

The identification of genes involved in the regulation of puberty is important for understanding the development and differentiation of sex-related brain function. In this study, we focused on the identification of genes of which expression is imprinted by treatment of estrogen during the critical period of brain sexual differentiation using the ddPCR approach. This method was proven useful to identify genes differentially regulated by certain treatments or physiological conditions (Liang and Pardee, 1992; Blanchard and Cousins, 1996; Berke et al., 1998; Huang et al., 1998). We identified 22 messages that are permanently modulated by neonatal treatment of estrogen. Twelve of them appeared to be novel sequences that were identified for the first time, whereas 10 showed 80-100% sequence homology with previously reported sequences in GenBank.

Northern blot analysis suggested that three of the messages identified by ddPCR could be involved in puberty initiation. Two messages of 2-1 and 8-1 genes showed sexually dimorphic patterns in the change of levels during postnatal development, therefore, might be involved in the sexual differences in the hypothalamus. KAP3 was confirmed to play an important role

in puberty initiation, since icv administration of antisene ODN clearly delayed vaginal opening.

The gene referred to as 8B was identified as KAP3, which is a KIF3A/3B associated protein. Recently, many kinesin superfamily proteins (KIFs) have been cloned and analyzed as a microtubule-based motor that transports membranous organelles anterogradely in cells, including neurons (Brady, 1985; Vale et al., 1985; Hirokawa et al., 1991; Hirokawa, 1993; Hirokawa, 1997). Various membranous organelles, such as precursors of the plasma membrane, synaptic vesicles, and mitochondria, are conveyed anterogradely. Among the members of KIFs, only the kinesin heavy chain and KIF3A/3B were known to have associated proteins. KAP3 was identified in the mouse brain and testis (Yamazaki et al., 1996) and was elucidated to be associated with KIF3A/3B at a ratio of 1:1:1 (KIF3A: KIF3B:KAP3). KAP3 was shown to bind to the tail domain of KIF3A/3B and to wrap around this small globular tail, but have no effect on the motor activity of KIF3A/3B (Yamazaki et al., 1996). Instead, it was believed to play a role in regulating the binding of KIF3A/3B to cargo.

As previously reviewed (Matsumoto, 1991; Leedom et al., 1994; Naftolin et al., 1996), estrogen can act as a neurotropic factor on neural tissues, stimulating axonal and dendritic growth and synapse formation during development. Estrogen has been known to have a facilitatory effect on axodendritic and axosomatic synapse formation in the deafferented arcuate nucleus in the adult female rat (Matsumoto and Arai, 1979; 1981; Matsumoto et al., 1985). Estrogen treatment to prepubertal rats also induced an acute increase of synaptic volume percent, area density (numbers of synapses/unit area of tissue section), and numerical density in the ARC (Clough and Rodriguez-Sierra, 1983). Subcutaneous administration of estrogen to prepubertal 25-day-old female rats induces accelerated synaptogenesis in the ARC concomitant with a precocious surge of the plasma luteinizing hormone (LH) at 1600 h on day 27 of age (Rodirguez-Sierra and Clough, 1987). The synaptic area densities of the ARC, medial preoptic area, and medial septal area are significantly higher on day 31 compared to day 27. In the cycling adult female rats, synaptic remodeling in the ARC during the estrous cycle is induced by estrogen and precedes the preovulatory gonadotropin surge (Naftolin et al., 1996).

The onset of puberty is also, at least in part, due to a stimulation of synapse formation by estrogen in the hypothalamus. The morphological change of GnRH secreting neurons was observed during juvenile development (Wray and Hoffman, 1986). During prepubertal development, as an animal grows, synaptic contacts are established among GnRH neurons themselves, as well as between GnRH cells and other neuronal systems (Wray and Hoffman, 1986; Urbanski and Ojeda, 1987). The onset of puberty may also be, in part, due to the retraction of inhibitory synapses between GnRH neuron and inhibitory neurotransmitter or neuromodulator such as the opioid peptide and GABA as previously reviewed (Ojeda and Urbanski, 1988; Becu-Villalobos and Libertun, 1995).

In this study, KAP3 mRNA level peaked just before the initiation of puberty and puberty was clearly delayed by icv injection of antisense KAP3 ODN. Therefore, it is presumed that KAP3 is involved in the molecular events prior to puberty initiation. Conclusively with previous reports, the results suggest that KAP3 may be involved in the processes of synaptic plasticity or synaptic remodeling before puberty. Although no data are available concerning the 5' flanking region of KAP3 and existence of estrogen response element, the present results strongly suggest that KAP3 is regulated by estrogen directly or indirectly through other factor(s).

Other genes of which messages were differentially regulated by neonatal treatment of estrogen and which showed increases before puberty initiation may play important roles in hypothalamic sexual differentiation and the onset of puberty. Although we have no direct evidence for the involvement of these gene products in sexual difference and puberty onset, these gene expressions were changed before the onset of female puberty, and confirmed to be responsive to the organizational effect of estrogen, according to the present ddPCR and northern blot analysis. Moreover, postnatal change patterns in mRNA levels of theses genes were different between sexes. Therefore, genes referred to as 2-1 and 8-1 may be involved in hypothalamic sexual differentiation and the onset of puberty. Our next step will be further analysis of these genes.

In summary, we identified several new estrogen responsive genes that may play important roles in puberty initiation and hypothalamic sexual differentiation.

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