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Ontogeny and Autoregulation of Androgen Receptor mRNA Expression in the Nervous System

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Androgens and the androgen receptor (AR) both play critical roles for the development of the male phenotype. To investigate the roles of androgens in the developing nervous system, we examined the AR messenger RNA distribution by *in situ* hybridization. Our results indicate that AR transcripts were detectable in male mouse embryos at embryonic day 11 (E11). Intensive AR labeling appears in the neuroepithelium of brain vesicles and spinal cord, as well as in the reproductive organs. During E15–E16, new and strong AR labeling appeared in the cortex of cerebrum and hippocampus. Specific AR signals were also present in the brain areas known for hormonal control of copulatory behavior and mediating sensory processing. Interestingly, many ganglia were found to express AR mRNA at E15–E16. These novel AR-expressing sites include the dorsal root, sympathetic, and celiac ganglia, as well as the opthalmic nerve of trigeminal ganglion. Sex dimorphism of AR expression in brain was also observed during E15–E16. Postnatally, brain and spinal cord can respond to circulating androgen levels by modifying their AR gene expression, but the ganglia cannot. Together, these data suggest androgens may have a great influence on the development and maintenance of the nervous system through the AR.

Key Words: Androgen receptor; embryogenesis; nervous system; autoregulation; *in situ* hybridization; RT-PCR quantitation.

Introduction

Testosterone (T) and its 5α-reduced metabolite, 5α-dihydrotestosterone, are the major circulating androgenic hormones in males. Both hormones bind to and activate the androgen receptor (AR) *(1)*. In addition, T can also be metabolized into estrogen by aromatase *(2)*. The role of estrogens in the development of the central nervous system (CNS) has been well documented *(3,4)*. These studies suggested that estrogens control a number of male typical brain functions and behaviors. In contrast, the androgenic effects via activation of the AR on the developing CNS are much less understood. Since circulating T can exert either estrogenic or androgenic action, the differential distribution and the ratio of the 5α-reductase vs aromatase or AR vs estrogen receptor (ER) in the target tissues may determine the potential action of T.

Many studies have been carried out to reveal the target and mechanism of androgenic actions in the adult CNS. AR mRNA distribution is high in regions known to be directly involved in endocrine feedback mechanism and reproduction. For example, the greatest densities of AR-positive cells were observed in the hypothalamus, and in regions of the telencephalon that provide strong inputs to the hypothalamus *(5)*. Such AR mRNA distribution in the brain is consistent with androgenic effects on the regulation of protein peptide production *(6)*, gonadotropin secretion *(7)*, and sex-specific behaviors *(8)*. In addition, high levels of AR mRNA were found in extrahypothalamic brain areas, such as the cortex, lateral septum, and hippocampus of the rat, which present the possibility that androgen action in the brain is not limited to reproductive activity *(9,10)*. The physiological significance of androgens in CNS has been studied in the whole animal or tissue-culture systems that were treated with androgens or antiandrogens, or were subjected to androgen deprivation. In general, these studies revealed clear androgenic effects on a wide variety of functions, including male sexual behavior, sex reflexes *(11)*, learning and memory tasks *(12)*, as well as motor neuron differentiation and regeneration *(13)*.

To know whether AR is present in the nervous system during embryogenesis, we examined AR mRNA distribution by *in situ* hybridization. Since AR ontogeny in reproductive organs is well documented, we used sagittal sectioning to include reproductive tissues as a positive control for each section. Surprisingly, our results show the presence of high levels of AR mRNA in the neuroepithelium of the mouse CNS during early development. Several

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novel AR mRNA-expressing ganglia were identified, and sex differences in AR expression among brain areas were observed during development. Furthermore, our data indicated that adult brain areas, but not ganglia, could respond to circulating androgen by altering their AR mRNA levels. Together, our data suggest AR could play critical roles in the development of the nervous system, and its expression could respond to the circulating androgen level in the postnatal stages.

Results

Overall AR mRNA Distribution Patterns During Mouse Embryogenesis

To examine the AR mRNA expression pattern during nervous system development of male mouse embryos, *in situ* hybridization was used. The gender of each embryo was determined by polymerase chain reaction (PCR) for the presence of Sry and Zfy genes (*see* Material and Methods). As shown in Fig. 1, AR transcripts could be detected as early as E11. The most intensive labeling was found over the neuroepithelia of the forebrain, hippocampus, and spinal cord as well as in the urogenital sinus and genital tubercle. As a negative control, sections hybridized with sense AR RNA probe did not detect any signal (data not shown).

During E13–E14, the labeling in several cortical regions becomes even stronger. Medium to low signals were detected in the hypothalamus and pituitary gland. AR expression could be detected in many peripheral tissues, such as the adrenal gland, kidney, genital tubercle, and perichondrium. Subsequently, at E15–E16, the AR signal intensity in the neocortex, cortex of hippocampus, and hypothalamus reached a maximum. Intensive AR expression could be detected in other brain regions, such as the olfactory bulb, peristriatum, preoptic area, and medulla oblongata, as well as nonneuronal tissues, such as the larynx, skin, and the perichondrium of certain bones (Fig. 1). High levels of AR mRNA in the nervous system declined toward the end of gestation, but expression in reproductive tissues remained strong (data not shown).

AR mRNA Distribution in the Developing CNS

It is known that sex differentiation in the mouse reaches a peak at E15–E16 *(14,15)*. When the E15 brain was examined under higher magnification, we observed AR transcripts located largely within the cell bodies of differentiated neuronal structures (Fig. 2). The strongest AR signals appear in the cortical plate and outer ventricular layer of the neocortex (Fig. 2C,D), the function of which is related to cognitive activity. In the midbrain, strong labeling was found in the superior colliculus and cerebellar primordium, suggesting that androgen may influence visuomotor activity (Fig. 2E,F). It is not surprising that the regions known to relay sensory information contained labeled neurons, e.g., in areas including the solitary tract, vestibular nucleus, olfactory bulb, and the nucleus of the lateral lemniscus (Fig. 2G–J). Medium density of labeling was detected in the ventromedial nucleus of the hypothalamus (Fig. 2G,H) and the preoptic area (Fig. 1), each of which is thought to play a key role in mediating the hormonal control of copulatory behavior. Periaqueductal gray neuroepithelium (Fig. 2E,F), which shares strong connections with hypothalamus *(16)*, displayed high AR expression as well. In addition, AR labeling could be detected in the dorsal horn of the spinal cord, which modulates the central relay of somatosensory information (Fig. 2I,J).

AR Expression in Peripheral Nervous System

Certain peripheral neural tissues at E14–E16 were able to be strongly labeled by AR antisense riboprobe. These tissues include the epithelia of nasal cavity and tongue (Fig. 1), as well as the sympathetic trunk, ophthalmic nerve of trigeminal ganglia, and the sympathetic, celiac, and dorsal root ganglia (Fig. 2K–P).

The ontogeny and distribution of AR transcripts in various neural tissues were summarized in Table 1. Some neural tissues transiently express AR, such as parastriatum, olfactory bulb, and celiac and sympathetic ganglia, whereas others continuously express AR once the expression was initiated. These data suggest that the AR expression level may control the sensitivity of neural tissues to androgen during development.

Sex Dimorphic Expression of AR mRNA in E16 Brain

To know whether AR expression pattern is different between sexes, we determined the sex for each embryo and compared their AR expression patterns. The most striking observation was that there are relatively low AR signals in the female brain as compared to the male brain (Fig. 1), whereas the AR signal intensities in peripheral organs of the female embryos, such as pituitary gland, larynx, kidney, adrenal gland, and external genitalia, were indistinguishable from those of the male embryos. This result indicates that gender-specific AR mRNA expression in brain areas occurs during a critical sex differentiation stage.

AR mRNA Expression in Adult Brain

Since the AR mRNA distribution in the adult mouse brain was well studied, we compared the AR mRNA expression pattern obtained with that published by Simerly et al. *(5)*. We showed that high density of AR labeling was detected in the CA1, CA2, and dentate gyrus, but not in the CA3 field of hippocampus. High to moderate AR signals were found in the piriform cortex, hypothalamus (VMH), arcuate nucleus, reticular nucleus of thalamus, and amygdaloid nucleus (Fig. 2Q,R). This AR mRNA distribution is consistent with previous reports *(5,10)* suggesting the hybridization signals observed were derived from AR mRNA.

Short-term Regulation of AR mRNA in Adult Neuronal Tissues

It has been shown that AR levels can be regulated by androgen. This phenomenon has been well demonstrated in

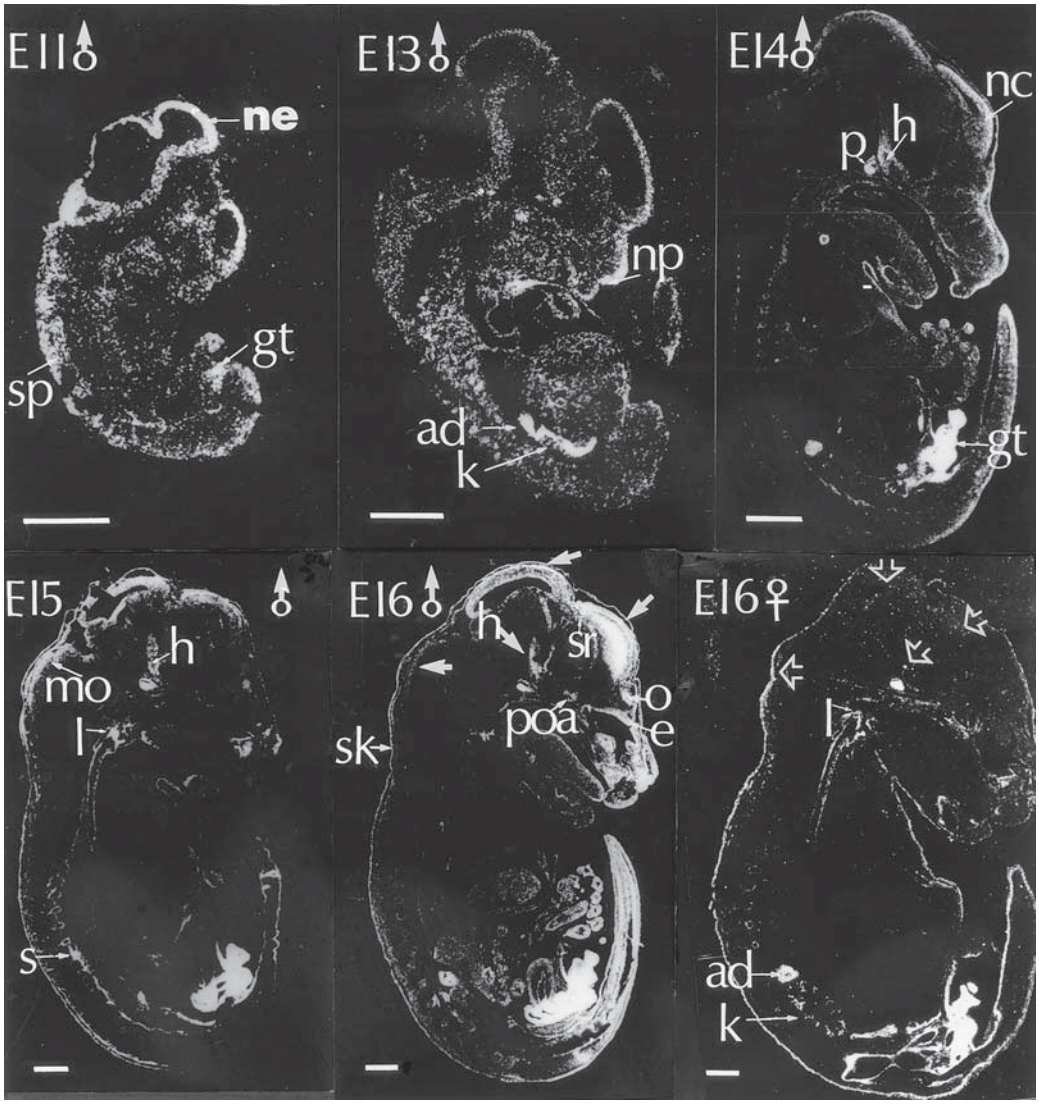

Fig. 1. Distribution of AR mRNA in mouse E11–E16 embryos examined by *in situ* hybridization. Slides including E11-E16 embryo sections were subject to *in situ* hybridization analysis in the same batch. Dark-field photomicrographs show the sagittal sections hybridized with [^{35}S]-labeled antisense AR RNA riboprobe. The bright areas in the dark-field pictures indicate the hybridized AR mRNA signals. The stage and gender of each embryo are marked in the upper-left corner of each photograph. Genital tubercle or urogenital sinus serves as the internal positive control. AR transcripts are present in various brain regions of the male embryo from E11 to E16, but not in many brain areas of E16 female embryos. In E16 female, black arrows indicate regions in which low AR signals were detected as compared to the strong signals indicated by white arrows in the E16 males. Abbreviations used are: ad, adrenal gland; e, olfactory epithelium; gt, geminal turbercle; h, hypothalamus; k, kidney; l, larynx; mo, medulla oblongata; nc, neocortex; ne, neuroepithelium; np, nasal process; o, olfactory lobe; p, pituitary gland; poa, preoptic area neuroepithelium; s, sympathetic trunk; sk, skin; sp, spinal cord; sr, stria medullaris. Size bar, 1 mm.

various reproductive organs, and we wondered whether it would also occur in neural tissues. Thus, we applied the quantitative reverse transcriptase (RT)-PCR assay to measure the AR mRNA level in various neural tissues. A titration reaction and its curve were used to assay and calculate the AR mRNA amount within the sample (Fig. 3A,B). Three days after castration, adult mice were injected with T over a period of 3 d. Animals with this treatment showed decreasing AR mRNA concentrations in most of the neuronal tissues examined in comparison to those in sham control animals. In contrast, AR mRNA levels tend to be increased in 3-d castrates. Among the neural tissues examined, hypothalamus expressed the highest AR mRNA (average around 2.8×10^8 copies/μg total RNA in intact mouse) and showed significant androgen responsiveness (Fig. 3C). The cerebellum and spinal cord also respond to androgen treatment, even though their AR mRNA levels were much lower. Cerebral cortex showed the same tendency as other brain areas examined. In contrast, the AR mRNA levels in the dorsal root and sympathetic ganglia did

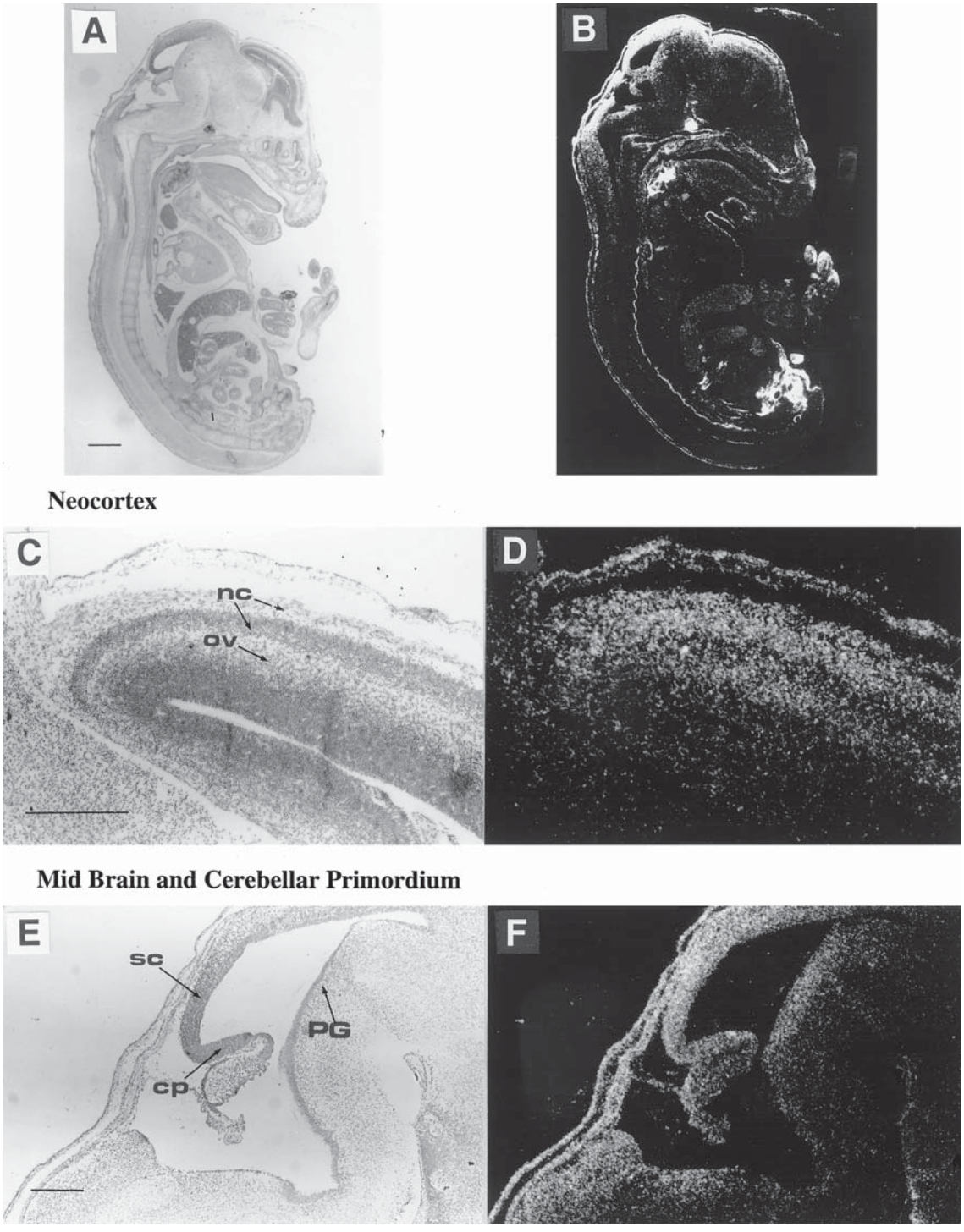

Fig. 2. *(pp. 82–84)* Distribution of AR mRNA in the nervous system of E15 **(A–P)** mouse embryo and in adult brain **(Q,R)**. Light-field photographs (left panel) reveal the tissue type of the same view in the dark-field photographs (right panel), which show the localization of AR transcripts. (A) and **(B)** show that the AR signal intensity in brain is comparable to that in the reproductive organs. The local tissue expressing AR mRNA is indicated on the top of each picture. Abbreviations used are: amg, amygdala; Arc, arcuate nucleus of hypothalamus; CA1-CA3, CA1-CA3 fields of the hippocampus; Cg, celiac ganglia; cp, cerebellar primordium; cx, cortex of hippocampus; dg, dentate gyrus; drg, dorsal root ganglia; DH, dorsal horn of spinal cord; LL, nuclei of lateral lemniscus; MVe, medial vestibular nucleus; nc, neocortex; on, ophthalmic nerve of trigeminal ganglion; ov, outer ventricular layer of cortical plate; p, pituitary gland; PG, periaqueductal gray; Pig, pigment epithelium of retina; Rt, reticular thalamic nucleus; s, sympathetic ganglia and sympathetic nerve trunk; UGS, urogenital sinus; VMH, ventromedial hypothalamic nucleus. Size bars represent 1 mm in (A) and 400 μm in (C)–(R).

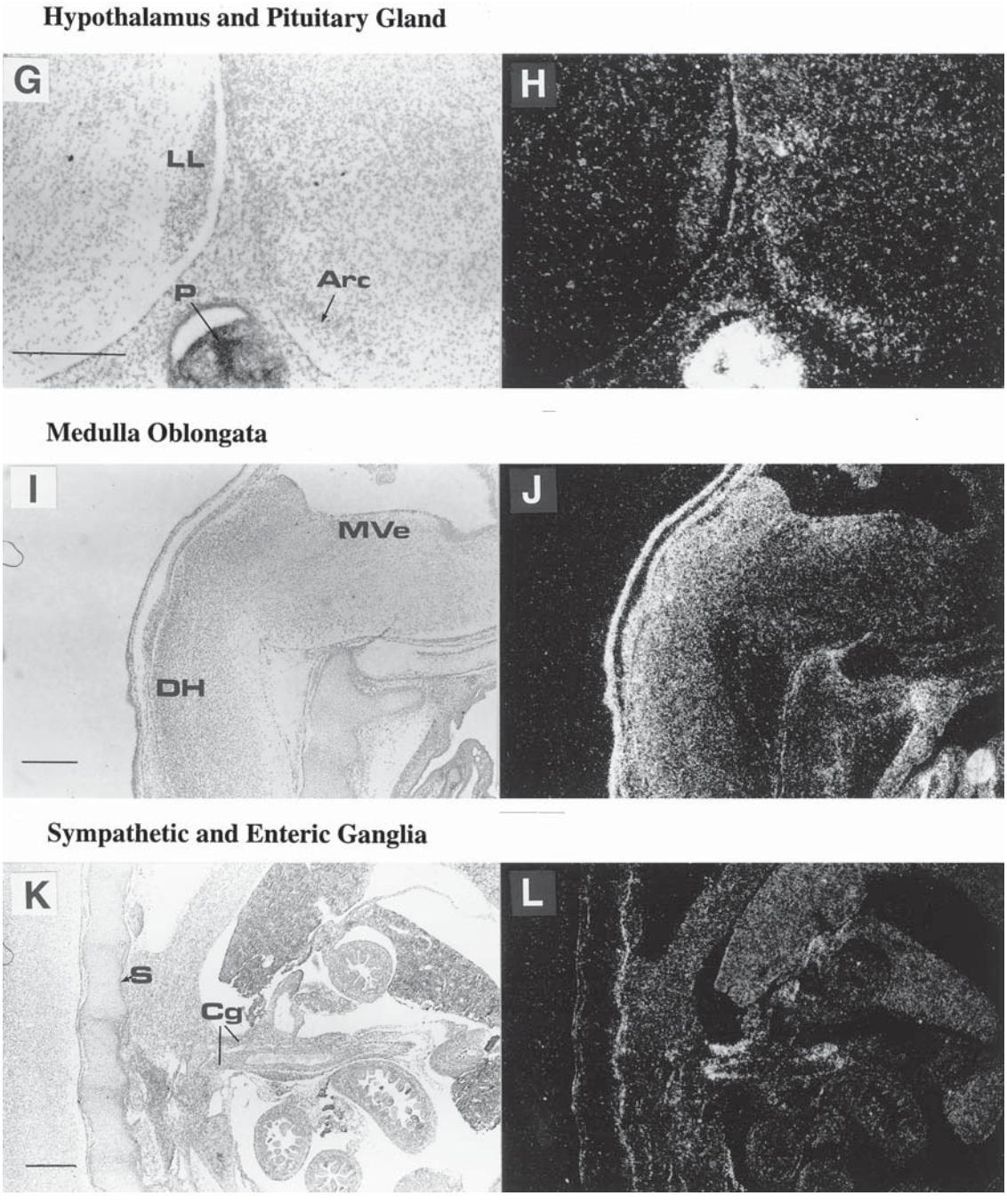

Fig. 2. *(continued)*

not show significant change with the androgen treatment. These data suggest that adult brain and spinal cord could respond to circulating androgens by changing their AR mRNA levels.

Discussion

In the present study, we provide novel overall AR mRNA expression patterns and identify several neural tissues with active AR mRNA expression during E11–E16. Our AR mRNA distribution data are consistent with the previous AR ontogeny studies in mammary gland *(17)*, prostate *(18)*, and other reproductive organs *(19)*, as well as in adult hippocampus *(10)*. However, these AR expression patterns did not follow those of other steroid receptors reported. Thus, we concluded that the AR mRNA distribution in the developing nervous system is specific, rather than crosshybridizing to other steroid receptors.

Systematic analysis of AR localization in the rodent nervous system during early prenatal stage has not been described. By using DNA-cellulose affinity chromatogra-

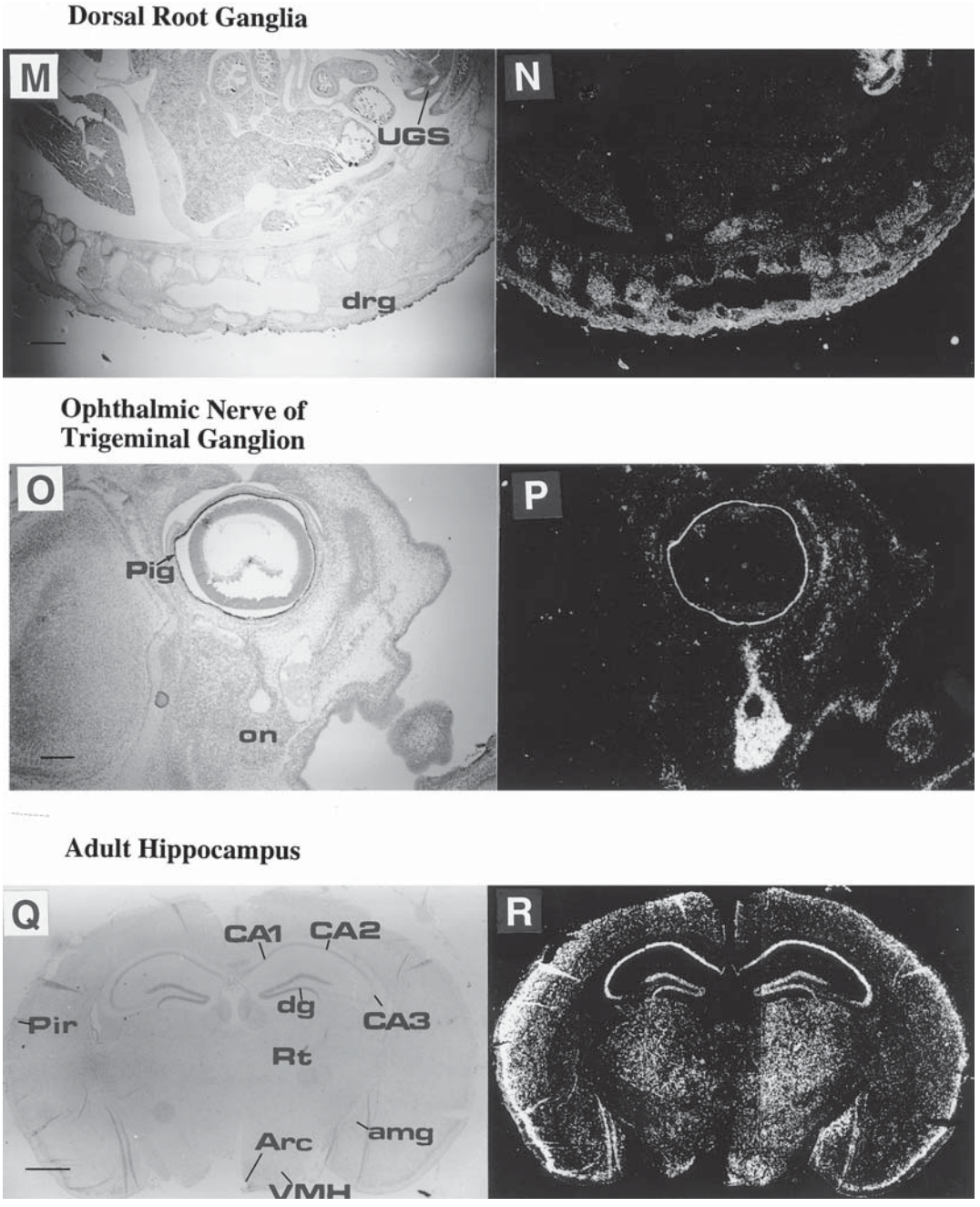

Fig. 2. *(continued)*

phy, Vito and Fox showed that an AR protein level that is capable of binding to [^{3}H]-DHT was significantly higher in hypothalamus-POA than other brain regions at E15–E16. Generally, the AR ontogeny profile revealed by Vito and Fox was consistent with our observation of the overall AR mRNA levels in brain *(20)*. Our data, however, showed AR mRNA appeared as early as E911. The expression was gradually increased and reached a peak at E15–E16. Other brain areas than the hypothalamus-POA also express a high level of AR mRNA. The differences between these two studies could be due to the sensitivity of methodology used or different expression kinetics between AR protein and mRNA. Since the most apparent prenatal sex difference exists at E15–E16 in mice, which correlates with the peak of serum androgen levels in males, these data support the hypothesis that the presence of AR in prenatal mouse brain may actually signal the onset of the critical period.

Our data showing positive AR expression in the spinal cord at E15 were in line with the findings that AR protein appears at E15, and androgen can directly regulate the development and morphology of the spinal genitofemoral nucleus *(21)*. In chicken, AR protein level within the hypo-

Table 1
Analysis of AR mRNA Expression in Selected Regions of the Developing Mouse Brain and Periphery[a]

Region	E11	E13	E14	E15	E16	E18
Olfactory cortex neuroepithelium	–	–	+	+	++	–
Cerebral cortex neuroepithelium	++	+	++	++	++++	++
Hippocampal cortex	++	+	++	+++	+++	+
Hypothalamus	–	–	+	++	++	+
Parastriatum	–	–	–	–	++	–
Medulla oblongata	+	+	+	+++	++	+
Brainstem	–	–	–	+++	++	+
Cerebellum	–	–	–	++	+	–
Spinal cord	+	+	+	+	+	+
Dorsal root ganglia	–	–	–	+	++	–
Sympathetic ganglia	–	–	+	+	–	–
Celic ganglia	–	–	+	+	–	–
Ophthalmic nerve of trigeminal ganglion	–	+	+	++	+	–
Nasal epithelium	–	–	–	+	+++	++
Pituitary gland	+	+	++	+++	++++	+++

[a]AR mRNA levels based on relative optical density values from photographs. Two pictures of each stages were used for scanning by Fotodyne image analysis system with Collage 4.0 software. Signal intensity was normalized to the intensity of the heart in each picture.

thalamus was detectable from E9.5 to E18.5, supporting the observation that T organizes masculine mating behavior prior to E13 in male chick embryo *(22)*.

Our data for an AR expression pattern highly overlap the expression pattern of 5α-reductase. According to the report published by Kerr et al. *(10)*, intensive 5α-reductase mRNA labeling was found in the proliferating neuroepithelium of the CNS and in spinal ganglia at E14. At E16–E18, 5α-reductase mRNA levels are highest in proliferating cerebral cortical, the basal telencephalic neuroepithelia, and the neuroepithelium of the amygdala. Differentiating parts of the basal telencephalon and the cortical plate were weakly labeled. During the neonatal stage, labeling in the cortical plate and thalamus increases even more *(23)*. Compared to our AR *in situ* data, this 5α-reductase mRNA distribution is similar to AR's, but the expression occurs somewhat later. Intensive 5α-reductase labeling was also detected in fetal liver and trigeminal ganglion *(23)*, but these tissues were negative for AR mRNA in our hand. Interestingly, 5α-reductase enzyme activity was found in neural structures rich in myelinated fibers *(24)*. This observation correlates with our finding of AR mRNA expression in the developing sympathetic trunk, supporting the idea that the white matter structures of the brain are a target for T action.

On the other hand, brain aromatase mRNA distribution and aromatase activity are expressed only in specific hypothalamic and limbic structures *(25)*. In the brain cortex areas, there was no detectable aromatase activity, but we could detect high levels of AR mRNA there. These data show a much stronger correlation of AR with 5α-reductase expression than AR with aromatase expression.

Most of the developing brain areas exhibiting high levels of AR mRNA were found to express AR in the adult, but the relative AR signal intensity among different brain regions varied with age. For example, in the adult rodent brain, the greatest densities of AR mRNA-labeled cells are located in the hypothalamus, and in regions of the telencephalon, including amygdala and amygdalo-hippocampal areas *(5)*. However, only moderate AR signals in hypothalamus and low AR signals in amygdala and amygdalo-hippocampal areas during midgestation stage were observed. In contrast, the strongest AR labeling was detected in the cortical plates of forebrain and midbrain throughout development. We also detected transient AR mRNA expression in certain developing ganglia and brain areas, indicating that the AR expression pattern may be altered throughout the mouse life-span.

This AR expression pattern overlaps with other steroid receptors in the developing CNS. Examination of ER distribution in the neonatal brain locates ER signals in the hypothalamus, preoptic area, amygdala, and cerebral cortex *(14,26)*, whereas glucocorticoid receptors (GR) are present before the time of birth throughout the brain; the highest density of GR labeling was found in the periventricular hypothalamus and in the CA1-CA2 pyramidal cell fields of the hippocampus. Moderate labeling was present in the anterior and posterior hypothalamus, subthalamic nuclei, and lateral septum. The GR pattern did not change significantly with age, with the exception of an increase in the intensity *(27)* . High densities of mineralocorticoid receptor (MR) mRNA-labeled cells were found throughout the hippocampal area. Strong MR labeling was found in layer II of the cortex, and modest MR labeling was found in the subornical organs and anterior hypothalamus *(28)*. Based on our *in situ* data, AR mRNA distribution is highly overlapped with ER's and slightly colocalized with other steroid receptors, indicating that these steroid receptors may work in concert to regulate brain development.

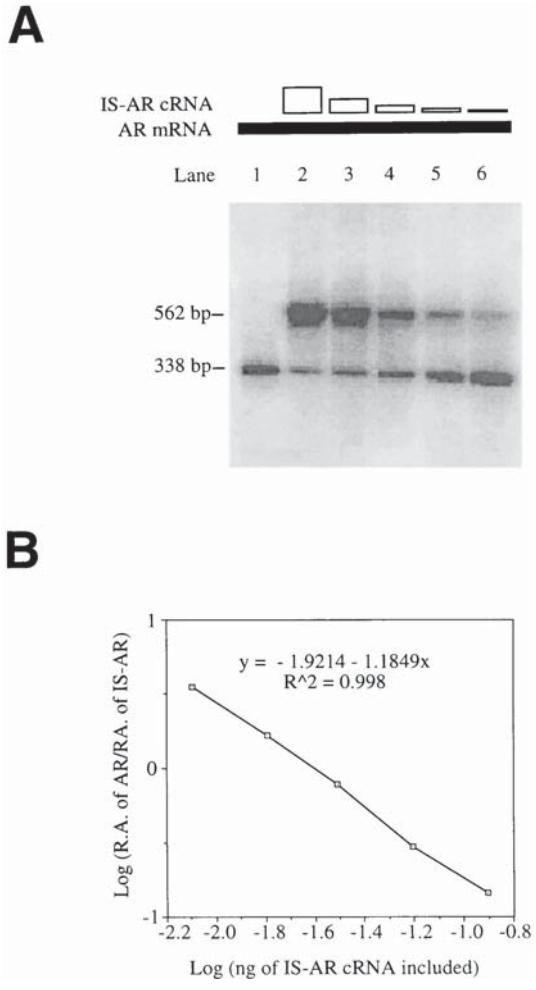

Fig. 3. Effects of castration and androgen replacement on the AR mRNA concentration in various neural tissues determined by quantitative RT-PCR. **(A)** An autoradiograph showing the quantitative RT-PCR assay for the AR mRNA level in the hypothalamus of an intact adult mouse. Total RNA (0.2 μg) was reverse-transcribed in the absence of (lane 1) or in the presence of two-fold diluted IS-AR RNA, ranging from 0.125 to 0.008 ng (0.275–0.0176 copies). The RT reaction mixture was subject to 26 cycles of PCR amplification, separated by electrophoresis, and visualized using a PhosphorImager. Sizes of the PCR products of AR and IS-AR are 338 and 562 bp, respectively. **(B)** The titration curve and linear regression analysis of (A). The radioactivity of each band was quantified by the Image Quant program. The log values of the intensity ratio of the AR band to the IS-AR band on the *y*-axis were plotted over the log values of the corresponding amounts of IS-AR RNA on the *x*-axis. The linear regression equation and the correlation coefficient (R^) of the linear curve are as shown. As calculated from the equation, the AR mRNA amount in the reaction equals the copy number of IS-AR (0.024 ng). Therefore, the AR mRNA concentration in the sample equals $0.024 \times 5 \times 2.2 \times 10^9$ copies/μg total RNA.

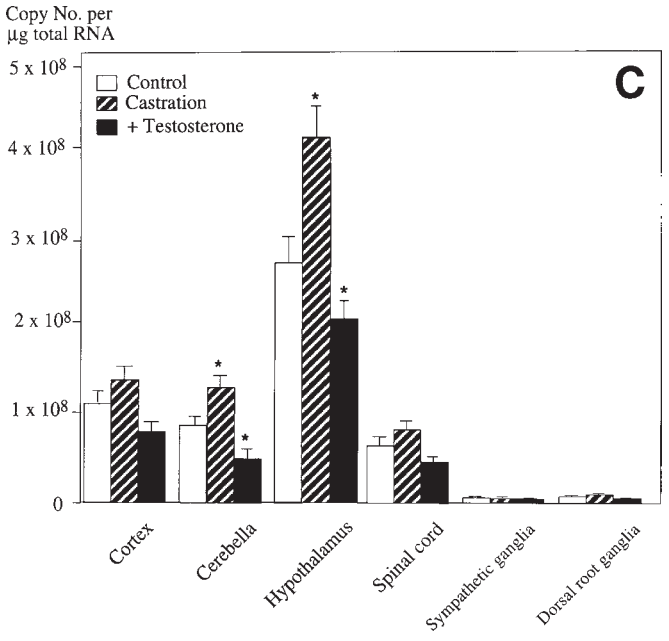

Fig. 3. *(continued)* Effects of castration and androgen replacement on the AR mRNA concentration in various neural tissues determined by quantitative RT-PCR. **(C)** Animals were shammed, bilaterally gonadectomized, and held for 3 d (castration), or castrated, held for 3 d, and then treated with T (0.5 mg/each/day) for 3 d. Total RNAs were individually isolated from the indicated tissues, and the AR mRNA contents were quantitated by competitive RT-PCR. Data are expressed as copy number per μg RNA. One-way analysis of variance revealed a significant effect of treatment. *P* value was significantly different from shamed control value (*$P < 0.05$) as determined by post-hoc analysis. Each bar represents the mean ±SEM of 3 determinations.

Sex differences in AR localization and in aromatase activity within adult rodent brain have been reported *(29)*. However, so far there is no report about the sex difference in the AR distribution in the developing brain. It is very interesting that our data showed the sexually dimorphic AR mRNA expression occurred in many brain areas of E16 mouse embryo. More information about the AR protein localization and the androgen responses in these brain areas of embryos will be essential to understand the significance of this observation.

Autoregulation of AR mRNA by its ligands has been reported in the reproductive organs, but is less understood in the neural tissues. Such regulation seems to be tissue- and time-dependent. Most previous studies examining AR mRNA regulation in peripheral tissues *(30,31)* and hypothalamus *(32)* have found that steady-state AR mRNA levels increase after castration and T treatment reversed the effect of castration; however, some discrepancies exist *(10)*. In contrast, castration of *tfm* rats and androgen treatment failed to regulate AR mRNA levels, suggesting such autoregulation requires a functional AR.

Our data indicated that AR mRNA levels in brain regions and the spinal cord could respond to androgen manipulation just like many peripheral organs respond. It has been reported that an antiandrogenic compound could also influence hippocampal AR mRNA concentration *(10)*. However, only a relatively small change (~25–50%) after short-term castration was observed. This magnitude of

change in AR mRNA level does not parallel the reported 2- to 10-fold induction of AR mRNA in rat peripheral tissues with similar treatment *(33)*. One of the possible explanations is that a relatively low level of AR is expressed by neural tissues compared to those in accessory sexual organs. In the brain regions, such as the hypothalamus and hippocampus, where fine-tuning of hormone feedback may be necessary, small and rapid changes in AR expression could have great functional significance. Further quantitative investigation of AR and the AR mRNA regulatory mechanism in discrete brain nuclei may be needed.

In the adult stage, AR activity is essential to maintain the normal function of spinal and bulbar motor neurons. When AR protein stability or transactivation ability were decreased in the case of expanded CAG repeats, patients with bulbospinal muscle degeneration syndrome (Kennedy's disease) were often presented *(34)*. Our data showed AR is expressed in the developing spinal cord. Then it will be interesting to know whether AR has an imprint effect on the later onset of Kennedy's disease. Although our data suggest a tendency for adult spinal cord to respond to circulating androgen, a bigger population needs to be tested to confirm the statistical significance of androgen responsiveness in spinal cord.

In summary, our studies showing specific spatio-temporal expression patterns of AR mRNA in the developing central and peripheral nervous system expand the role for AR during sex differentiation of the brain. Hormone manipulation in the adult can change the AR mRNA level, which may have a profound influence on the neuronal activities and expression of behavior.

Materials and Methods

Animals

Mouse embryos from C57BL/J (Jackson Laboratories, Bar Harbor, ME) were collected between E9 and E18 for *in situ* hybridization analysis. For androgen response experiments, young (2-mo-old) adult male mice (C57BL/J) were maintained on a 12-h light, 12-h dark cycle and given free access to food and water. For *in situ* hybridization study, the embryo numbers used were as following: E11, 7; E13, 3; E14, 10; E15, 6; E16, 5; E18; 5. Eighteen to parasaggital sections, which cover left, middle, and right panels, were examined. Bilateral gonadectomies were performed under ether anesthesia, and all animals were killed by decapitation. Brain dissections were performed as previously described *(35)*.

Sexing Embryos by PCR

Detection of Sry and Zfy genes, which present on the Y chromosome, was adapted for sexing early embryos using PCR *(36)*. Genomic DNAs were isolated from embryo sections and were PCR-amplified with the Sry- and Zfy-specific primers. The oligonucleotides used were as follows:

Sry 2: 5'-TCTTAAACTCTGAAGAAGAGA-3'
Sry 4: 5'-GTCTTGCCTGTATGTGATGG-3'
Zfy 3: 5'-AAGATAAGCTTACATAATCACATGGA-3'
Zfy 4: 5'-CCTATGAAATCCTTTGCTGCACATGT-3'

Probe Preparation

The cDNA fragment encoding the human AR ligand binding domain was subcloned into pBluescript SK+ (Stratagene, La Jolla, CA). [^{35}S-UTP]-labeled antisense and sense RNA probes were synthesized using T3 or T7 RNA polymerase, according to manufacturer's conditions (Ambion, Austin, TX). These probes were partially degraded to 150–300 bp by limited alkaline hydrolysis. Unincorporated nucleotides were removed by chromatography through a Sephadex G-50 column (Pharmacia, Piscataway, NJ).

In Situ *Hybridization*

Embryos were fixed in 4% paraformaldehyde in PBS, dehydrated in ethanol, cleared with xylene, and embedded in paraffin. Serial sections (6–8 μm) were collected on poly-L-lysine-coated slides, air-dried, and stored at 4°C under desiccation.

In situ hybridization was performed as described previously *(37)*. Briefly, the sections were deparaffinized, hydrated, and then treated with proteinase K (Boehringer Mannheim, Indianapolis, IN) at 0.2 μg/mL for 7.5 min. After washing in PBS, the sections were fixed in 4% paraformaldehyde, acetylated, dehydrated, and air-dried. The sections were hybridized at 50°C for 17 h with cRNA probes (the specific activity for both sense- and antisense probes reached 10^9 cpm/μg), 10^6 cpm of probe/slide in 50% formamide. Washes were performed with high stringency (50% formamide) at 65°C both before and after RNase digestion (20 μg/mL). The slides were dipped into Kodak NTB2 emulsion, and were exposed for 2 wk for dark-field analysis unless otherwise mentioned. When the time was due, slides were developed in Kodak D19 developer, fixed, and counterstained with hematoxylin.

Competitive RT-PCR Quantitation

AR mRNA quantitation essentially follows the method as described previously *(38)*. The internal standard plasmid used was called T7-AR-IS, which contains a exogenous DNA fragment (a 224-bp *Nco*I fragment from pSV-β-galactosidase; Promega, Madison, WI) within the AR-BC fragment (AR cDNA nucleotide sequence 2512–2849, which is amplified by PCR using AR-B and AR-C primers). The T7-AR-IS plasmid was linearized by *Ssp*I and transcribed by T7 RNA polymerase according to manufacturer's protocol (Ambion). The *in vitro*-transcribed RNA, IS-AR cRNA, was further purified by phenol/chloroform extraction and quantitated by absorbance at 260 nm.

To determine the AR mRNA amount, one titration reaction was run for each RNA sample. Total RNA (0.2 μg) was reverse-transcribed with five sets of twofold serial diluted IS-AR cRNA and then PCR amplified for 26–28 PCR

cycles using [^{32}P]-radiolabeled AR-specific primers (AR-B: 5'-TCACACATTGAAGGCTATGAA-3'; AR-C: 5'-CGGTACTCATTGAAAACCAG-3'). The PCR program consists of 1 min at 95°C and 1 min at 60°C.

PCR reaction products (10 μL) were separated on a 1.5% agarose gel, which was then dried and visualized by PhosphorImager Scanner. The radioactivity of each band was quantified by Image Quant Software (Molecular Dynamics, Sunnyvale, CA).

To calculate the AR mRNA level in samples, the intensity ratio of the AR band to the IS-AR band vs a known amount of included IS-AR RNA was plotted on a log scale. Five points should form a line. The correlation coefficient (R^) and the linear regression curve derived were used to monitor the quality of RT-PCR quantitation. Only when the correlation coefficient was higher than 0.98, the linear regression equation was used. The AR mRNA amount in the reaction is equal to the *x*-intercept value where the *y*-value is 1 (the equivalent point of AR and the known amount of included IS-AR). The AR mRNA copy number per μg of total RNA = the number obtained from the titration curve × 2.2 × 10^9 (copies/ ng) × 5 (dilution factor)

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