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Characterization of aromatase cytochrome P-450 mRNA in rat perinatal brain, ovary, and a Leydig tumor cell line: evidence for the existence of brain specific aromatase transcripts

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The conversion of androgens to estrogens is catalyzed by the aromatase cytochrome P-450 (P-450$_{AROM}$) in a variety of tissues and cell types in vertebrates. The manner in which aromatase activity is regulated appears to be quite different, even between different tissues of a single species. In the current study, we have determined the sequence of the 5′ end of the aromatase mRNA in a rat Leydig tumor cell line, R2C, and in three rat tissues [adult ovary, perinatal amygdala (AmY), and medial basal hypothalamus-preoptic area (MBH-POA)], each of which shows different patterns of aromatase expression. S_1 nuclease protection and primer-extension analyses establish that the site of transcription initiation of the aromatase mRNA present in rat ovary and the rat Leydig tumor cell line R2C is located approximately 97 nucleotides upstream from the initiator methionine. By contrast, although aromatase mRNA was detected in S_1 nuclease protection experiments using a probe derived from the aromatase open-reading frame, transcripts initiating at this site were absent from RNA samples prepared from perinatal rat AMY and MBH-POA tissue. S_1 mapping and sequencing of the 5′ end of AMY and MBH-POA aromatase cDNAs indicate that the aromatase mRNA transcripts present in these rat neural tissues from perinatal animals contain a distinctive 5′ terminus and are derived from a different promoter.

Keywords: rat; aromatase; development; brain; ovary

Introduction

The biosynthesis of estrogens from androgens is catalyzed by a specific microsomal cytochrome P-450 enzyme termed aromatase cytochrome P-450 (P-450$_{AROM}$) (Corbin *et al.*, 1988). Estrogens are also formed in a variety of peripheral tissues and cell types, in which p-450$_{AROM}$ expression is regulated in a differential fashion. For example, in ovarian tissue, aromatase is stimulated by cyclic AMP (Steinkampf *et al.*, 1988). Conversely, in the rat Leydig cell tumor line (R2C) and brain explants, P-450$_{AROM}$ activity is inhibited by activation of adenylate cyclase (Lephart *et al.*, 1990, 1992). Furthermore, recent evidence suggests that the tissue-specific regulation of the human P-450$_{AROM}$ gene is due, in part, to utilization of tissue-specific promoters in adipose (Mahendroo *et al.*, 1991, 1993), ovary (Means *et al.*, 1991) and placental tissues (Kilgore *et al.*, 1992). Finally, P-450$_{AROM}$ gene expression in the chicken ovary appears to utilize a single promoter localized immediately 5′ to the first coding exon, whereas a second promoter is active in the ovary and extragonadal tissues of Sebright and Campine birds (Matsumine *et al.*, 1991).

It is well established that the central nervous system is able to aromatize androgens to estrogens (Naftolin *et al.*, 1975; Callard *et al.*, 1978a,b). Estrogens formed locally in specific brain areas are thought to be involved in the sexual differentiation of neural structures perinatally (Toran-Allerand *et al.*, 1983; Gorski, 1989) that in turn establish the functional regulation of gonadotropin secretion patterns and sexual behavior (McEwen *et al.*, 1977; Parsons *et al.*, 1984). During postnatal development androgens appear to regulate the basal levels of aromatase activity in discrete nuclei of the preoptic area (POA) and medial basal hypothalamus (MBH) (Roselli & Resko, 1993). However, the activity is not altered by androgens in the amygdala (AMY) (Roselli & Resko, 1993), suggesting that different mechanisms may operate to regulate the expression of P-450$_{AROM}$ at different sites within the central nervous system.

In this study, we examined the basis of these different patterns of P-450$_{AROM}$ expression in the rat by characterizing the 5′ terminus of the aromatase mRNA that is expressed in a rat Leydig tumor cell line (R2C) and three rat tissues (adult ovary and the AMY and MBH-POA of perinatal animals). Our study indicates that at least two distinct mechanisms exist for the regulation of aromatase expression in rat.

Results

To initiate studies of the *cis* elements controlling aromatase expression in extragonadal (brain) and gonadal tissues of the rat, we isolated genomic DNA fragments encoding the 5′ flanking segments of the rat aromatase gene. As shown in Figure 1, the initiator methionine is preceded immediately by segments that closely correspond to a TATA and a CAAT box. The sequence of our genomic clone agrees with that observed by Hickey *et al.* (1990) except for a region of 44 nucleotides (shown in brackets in Figure 1) that was present in our genomic clone but not in the previously reported nucleotide sequence (Hickey *et al.*, 1990). As such, the structure of this genomic clone agrees with the structures of the genomic clones reported by Fitz-

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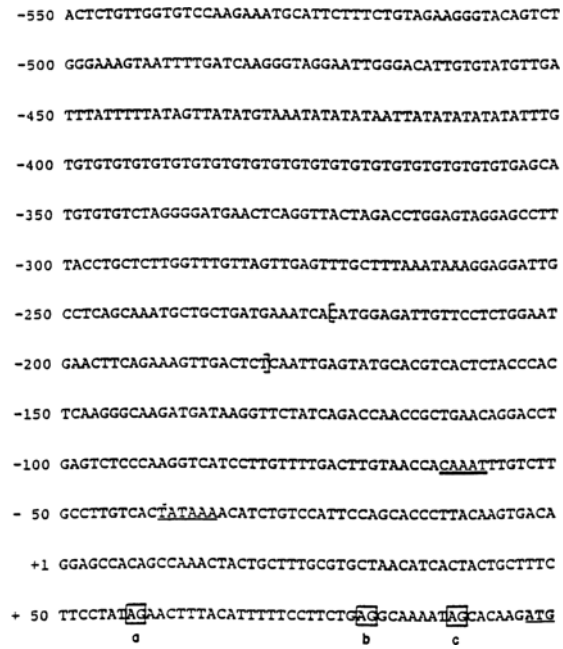

Figure 1 Nucleotide sequence of genomic DNA encoding the 5′ flanking region of the rat aromatase gene. A 1.5 kb PstI restriction endonuclease fragment (see Figure 7) was isolated from a rat genomic DNA clone approximately 15 kb in length. The site of transcription initiation (indicated by + 1) of the aromatase gene is located approximately 40 nucleotides downstream from a TATA box motif (TATAAA), 62 nucleotides from a CAAT box (CAAAT) and 97 nucleotides upstream from the initiator methionine (ATG). The region of 44 nucleotides (shown in brackets) was not contained in the sequence reported by Hickey *et al.* (1990), but corresponds to the sequences reported by Fitzpatrick & Richards (1993) and Lynch *et al.* (1993). The notations 'a', 'b' and 'c' refer to the AG dinucleotides of potential splice acceptor sites. The boxed AG dinucleotide 'b' (at nts = + 80–81) corresponds to the acceptor splice site utilized in the extragonadal expression of aromatase mRNA in the Sebright chicken (Matsumine *et al.*, 1991)

patrick & Richards (1993) and Lynch *et al.* (1993). The organization of a 1.5 Kb PstI endonuclease fragment is shown schematically in Figure 2.

The lack of uniform regulation of aromatase activity in various rat tissues and cell lines by cyclic AMP (Steinkampf *et al.*, 1988; Lephart *et al.*, 1990, 1992) prompted us to determine whether in the rat multiple promoters are utilized for P-450$_{AROM}$ expression. To characterize the site of transcription initiation, primer-extension experiments were performed using an oligonucleotide (shown in Figure 2C) to prime first strand synthesis using RNA samples prepared from fetal rat brain, a rat Leydig cell tumor line (R2C) or ovarian tissue. As can be inferred from results shown in Figure 3, the site of transcription initiation of the aromatase gene is located approximately 40 nucleotides downstream from a TATA box motif, 62 nucleotides from a CAAT box and approximately 97 nucleotides upstream from the initiator methionine in RNA prepared from rat ovary. Furthermore, as evident from Figure 3, these experiments also suggest that the same site of transcription initiation is used in the R2C cell line. No primer-extended product could be visualized consistently in our primer-extension experiments using RNA prepared from hypothalamic and preoptic area (MBH-POA) tissue from fetal or adult rats.

The results from the primer-extension analysis suggest that the site of transcription initiation utilized in ovarian tissue and in the R2C cell line is 97 nucleotides upstream from the initiator methionine. To confirm this result, S_1 nuclease protection analysis was performed using RNA prepared from rat tissues and the R2C cell line that expresses P-450$_{AROM}$. A uniformly labeled RNA probe 388 nucleotides in length was employed which spans the putative transcription initiation sites (− 144 to + 244 shown as B in Figure 2). In these experiments a protected band of 244 nucleotides in length was detected in RNA samples (10 μg total RNA) prepared from rat ovary and the R2C cell line. No signal was detected in RNA samples (10 μg total RNA) prepared from rat liver or placenta, while the lane containing tRNA served as negative control (Figure 4). Although these experiments identified a small proportion of transcripts that appeared to initiate 10–30 nucleotides upstream in R2C cells, these results confirmed that the principal site of transcription initiation is similar in the R2C cell line and ovary.

In the analyses of the ovary and R2C RNA samples, the S_1 nuclease experiments gave consistent results that were in agreement with the results of the primer-extension experiments. In the analysis of the rat brain RNA samples, however, primer-extended products could not be detected and the S_1 nuclease experiments yielded inconsistent results (data not shown), as the 244 nucleotide protected fragment identified in rat ovary and R2C samples (Figure 4) could not be detected when RNA samples prepared from neural tissue were tested, although previous studies (via Northern analysis) have shown aromatase mRNA to be present during this time (Lephart *et al.*, 1992a).

To examine the possible explanations for this discrepancy, we performed S_1 nuclease protection assays on samples of RNA prepared from perinatal amygdala (AMY) and MBH-POA tissue using two different probes: one spanning the site of ovarian aromatase mRNA transcription initiation (termed the 'ovarian promoter' probe derived from plasmid BSrAromPromI; see Figure 2B) and a second encoding the amino terminal segment of the aromatase P-450$_{AROM}$ open-reading frame (termed the 'coding region' probe derived from plasmid BSrArom4845; see Figure 2D and E). Several conclusions can be derived from the results depicted in Figures 5 and 6. First, consistent with the experiments summarized in Figures 3 and 4, the promoter immediately 5′ to the aromatase coding segment is used principally in ovarian and R2C RNA samples. Furthermore, when the R2C and ovarian RNA samples are analysed, the relative abundances of the protected bands are similar using both the 'coding region' probe and the 'ovarian promoter' probe. These findings contrast sharply with the results obtained when samples of RNA from rat neural tissues are analysed. Although, aromatase mRNA is detected in the AMY and MBH-POA specimens using the ('coding region') probe (Figure 5), no aromatase transcripts can be detected that are initiated at the 'ovarian' promoter (Figure 6). Instead, a partially protected band is visualized in MBH-POA and AMY (indicated by the arrowhead in Figure 6). The size of this partially protected fragment would correspond to the use of one of the three potential AG dinucleotide boxed in Figure 1 as a splice acceptor site(s). This same protected frag-

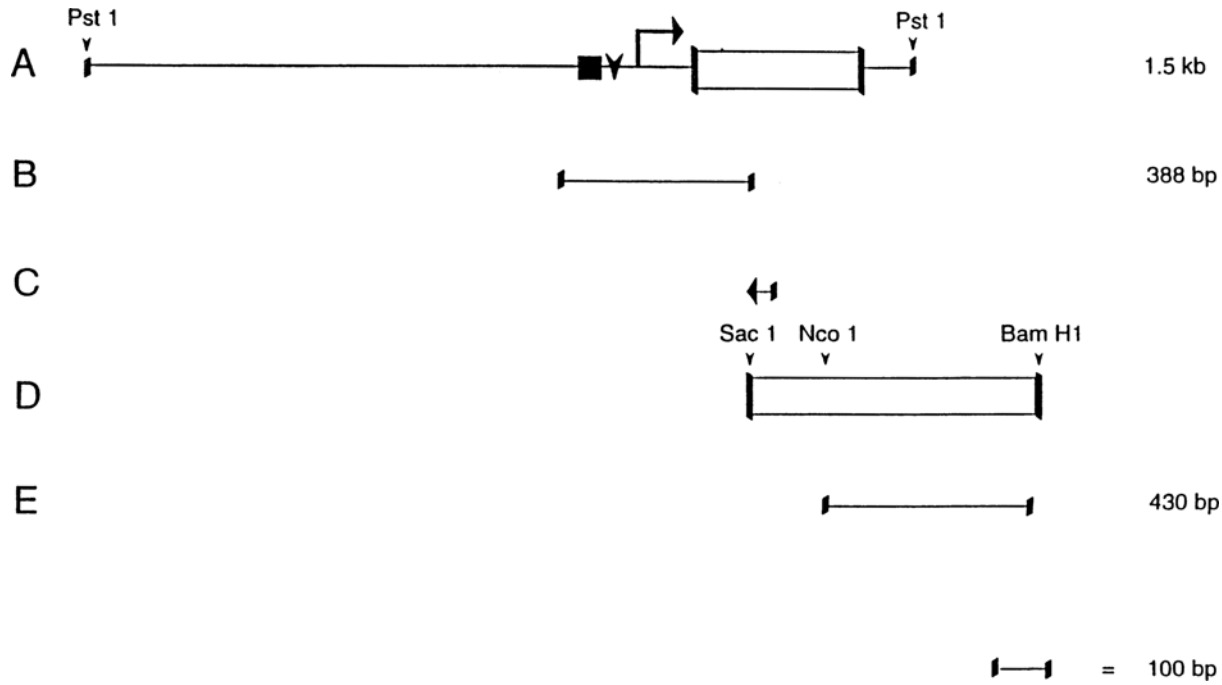

Figure 2 (**A**) Rat P-450$_{AROM}$ genomic clone. This PstI restriction endonuclease fragment contained a portion of the coding region (shown by the boxed rectangle) and the 5′ flanking region sequence of the rat P-450$_{AROM}$ gene. The right-hand arrow indicates the site for initiation of transcription in rat ovary and R2C cells (i.e., approximately 97 nucleotides upstream from the initiator methionine). The black box indicates a CAAT box motif while the black triangle denotes a TATA box. (**B**) Schematic of the position of the hybridization probe which spans the 'ovarian' promoter. A plasmid containing the exonic segment and the 5′ flanking region of the rat P-450$_{AROM}$ gene (as shown in **A**) was derived as described in the Materials and methods from the plasmid BSrAromPromI and utilized as the 'ovarian promoter' probe in the S_1 nuclease protection assays. (**C**) Indicates the location of the oligonucleotide used for primer-extension experiments. An oligonucleotide (5′-ACAAGCTTACCTGGTATGGAAGATG-AGCTCTCAC-3′) that overlaps with the 3′ sequence of the probe shown in (**B**) was utilized as primer in the primer-extension studies. (**D**) Shows the relative position of the hybridization probed used to detect RNA encoding the amino terminal segment of the aromatase open-reading frame. A plasmid containing the indicated segment of the rat P-450$_{AROM}$ gene (plasmid BSrArom4845) was utilized as the 'coding region' probe in the S_1 nuclease protection assays. (**E**) Shows the expected size of the protected fragment when the 'coding' probe was used in the S_1 nuclease protection analysis (as shown in **D**)

ment appears as a minor band in RNA prepared from ovary and R2C cells but not in the RNA samples containing liver RNA or tRNA when either probe was used.

These experiments suggest that the aromatase mRNA detected in perinatal AMY and MBH-POA tissue differs in the structure of its 5′ terminus. The presence of a partially protected band of approximately 170 nucleotides in RNA specimens prepared from the MBH-POA and AMY is consistent with transcription of aromatase mRNA from a distinct promoter followed by splicing of these transcripts to form aromatase mRNA that has a distinctive 5′ terminus.

To examine this possibility, we utilized the technique of rapid amplification of cDNA ends (RACE) to prepare primer-extended cDNA libraries using RNA from perinatal AMY and MBH-POA tissue. Six independent recombinants from AMY library and two independent recombinants were analysed and the nucleotide sequences summarized in Figure 7. The nucleotide sequence of these clones provide direct evidence that P-450$_{AROM}$ mRNA expressed in AMY and MBH-POA tissue isolated from perinatal rats contain a different 5′ terminus compared to that detected in rat ovary and R2C cells. Comparison of these sequences with the sequence of the 5′ flanking segment of the rat aromatase gene presented in Figure 1 reveals that the AMY and MBH-POA P-450$_{AROM}$ mRNAs are products of a distinct promoter which is processed to yield an aromatase mRNA that contains a distinct 5′ terminus. The nucleotide sequences from both the AMY and MBH-POA primer-extended clones indicate that a single 5′ acceptor splice site (designated by the boxed AG dinucleotide in Figure 7) is utilized for the differential processing of P-450$_{AROM}$ mRNA in both of these neural structures.

Discussion

While estrogen is formed in a variety of tissues, P-450$_{AROM}$ expression appears to be controlled by different mechanisms in different cell types. In some, such as the ovary, P-450$_{AROM}$ activity is stimulated by cyclic AMP, whereas, P-450$_{AROM}$ activity is inhibited by activation of adenyl cyclase in the rat Leydig cell tumor line (R2C) and in explants of perinatal rat brain tissue (Lephart *et al.*, 1990, 1992). Furthermore, basal levels of P-450$_{AROM}$ activity are regulated by androgens in medial basal hypothalamic (MBH) and preoptic area (POA), but not in amygdaloid (AMY), tissue during postnatal development (Roselli & Resko, 1993). Finally, previous evidence exists demonstrating tissue-specific regulation of the human and chicken P-450$_{AROM}$ gene is due to utilization of tissue-specific promoters (Mahendroo *et al.*, 1991, 1993; Matsumine *et al.*, 1991;

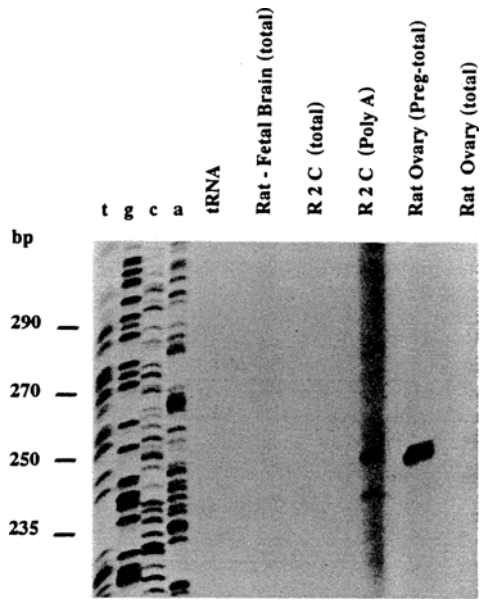

Figure 3 Primer-extension analysis of the transcription initiation site of the rat P-450$_{AROM}$ gene in different tissues. Fetal rat brain [medial basal hypothalamic and preoptic area tissue (MBH-POA); 10 μg total RNA], rat Leydig cell tumor line (R2C; 10 μg total RNA or 5 μg Poly A RNA) or ovarian tissue obtained during gestation (10 μg total RNA) or from random cycling animals (10 μg total RNA) were hybridized to an end-labeled primer corresponding to bases + 219 to + 244 relative to the P-450$_{AROM}$ translation start site and extended with avian myeloblastosis virus reverse transcriptase. The products were electrophoresed on a denaturing acrylamide gel, dried, and autoradiographed. The lane containing tRNA served as negative control. A sequencing ladder, shown to the left, was included to determine sizes from the sequencing reactions of the rat P-450$_{AROM}$ promoter template using the same oligonucleotide primer, as shown in Figure 2C. A similar primer-extended band (252 nucleotides in length) is visible in the R2C and ovary primer-extension experiments. This band is not visible in the tRNA or brain RNA lanes. The shorter fragment at ≈240 nucleotides is believed to be the result of nonspecific hybridization, as it is detected in samples of RNA that do not express aromatase mRNA (data not shown)

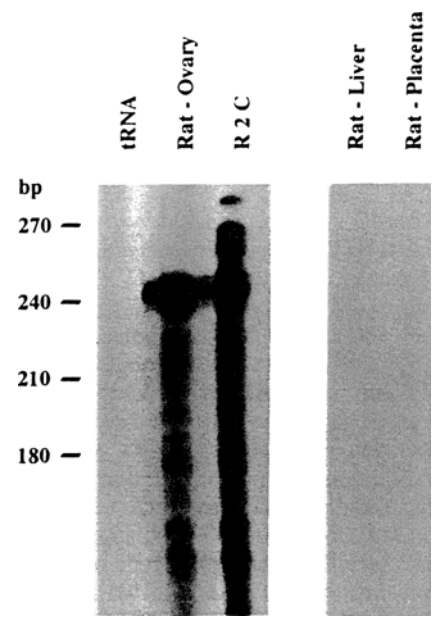

Figure 4 S_1 nuclease protection analysis of total RNA from rat ovary, a rat Leydig tumor cell line (R2C), rat liver, and rat placenta. RNA samples (10 μg) were hybridized to a uniformly labeled RNA probe 388 nucleotides in length which spans the putative transcription initiation sites (− 144 to + 244; shown as probe B in Figure 2). After hybridization, the samples were digested with S_1 nuclease, precipitated, and electrophoresed on a denaturing acrylamide gel and visualized by autoradiography. The positions of the size markers are shown to the left. The lane containing tRNA served as negative control. While aromatase activity has been demonstrated in human liver (Smuk & Schwers, 1977) and placental tissue (Siiteri & MacDonald, 1966), P-450$_{AROM}$ mRNA was not detectable in these tissues in rat

Means *et al.*, 1991; Kilgore *et al.*, 1992; Lephart *et al.*, 1992a).

The findings of the current studies provide insight into the regulation of the rat P-450$_{AROM}$ gene. Based upon the primer-extension and S_1 nuclease mapping analyses, the results of the present study suggest that although P-450$_{AROM}$ enzymatic activity is regulated in opposite directions by cyclic AMP in rat ovary and in the rat Leydig cell line R2C (Steinkampf *et al.*, 1988; Lephart *et al.*, 1990, 1992), P-450$_{AROM}$ RNA transcription is initiated in the ovary and R2C cell line at similar sites. Our results indicate that the principal site for initiation of transcription of the aromatase gene in both instances is 97 nucleotides 5′ to the initiator methionine and approximately 40 and 62 nucleotides 3′ to TATA and CAAT box motifs, respectively, in agreement with the recent reports (Fitzpatrick & Richards, 1993; Lynch *et al.*, 1993). This finding is also in agreement with previous analyses of the promoter that is active in human (Means *et al.*, 1991; Toda *et al.*, 1994) and chicken ovary (Matsumine *et al.*, 1991). Thus, our data suggest that in the rat a single promoter is utilized to effect the very different patterns of aromatase expression evident in the ovary and R2C cell lines (Steinkampf *et al.*, 1988; Lephart *et al.*, 1990). It is possible that the minor longer protected bands detected in S_1 nuclease experiments using R2C RNA may reflect differences in the composition of transcription factors controlling aromatase expression in the R2C and ovarian granulosa cells.

While the data presented demonstrate that the different patterns of aromatase regulation in the rat ovary and R2C cells utilize the same promoter, our data indicate that a different mechanism controls aromatase expression in the developing perinatal rat brain. We have previously determined in MBH-POA tissue during perinatal development that P-450$_{AROM}$ enzymatic and mRNA levels peak at gestational days 19 to 20 (Lephart *et al.*, 1992a). Furthermore, P-450$_{AROM}$ mRNA has been detected in MBH-POA and AMY areas by *in situ* hybridization during perinatal development (Lauber *et al.*, 1993). In RNA preparations from MBH-POA and AMY tissues of late gestational ages, transcripts initiating at the 'ovarian' promoter could not be detected. This negative result in specimens that we have shown contain aromatase mRNA (Lephart *et al.*, 1992a) led us to examine the levels of aromatase mRNA abundance in these specimens using two probes, one spanning the 'ovarian' promoter ('ovarian promoter probe') and a second encompassing a segment of the amino terminus of the aromatase open-reading frame ('coding region probe'). These experiments confirmed the presence of aromatase mRNA in these specimens and suggested that a majority of aromatase mRNA detected in RNA prepared from MBH-POA and AMY tissue from fetal

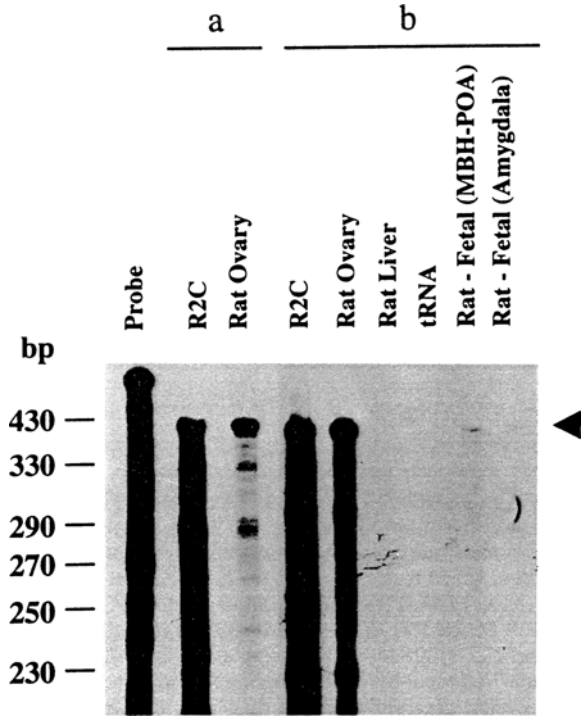

Figure 5 S_1 Nuclease protection analysis of total RNA from a rat Leydig tumor cell line (R2C), rat ovary, rat liver, and rat fetal brain [medial basal hypothalamic and preoptic area (MBH-POA) and amygdala (AMY)] using a probe complementary to a segment of the aromatase open-reading frame. The RNA samples (10 μg) were hybridized to a uniformly labeled probe encoding the amino terminal segment of the aromatase open-reading frame [nucleotides 262 to 692 (Lephart *et al.*, 1990), see Figure 2D and E]. The size markers are shown at the left (based upon a sequencing ladder that was utilized as reference but not shown in this exposure). (**a**) Indicates a short exposure (2 days) of the R2C and rat ovary mRNA samples. (**b**) Indicates a long exposure (5 days) of all mRNA samples. The lane containing tRNA served as negative control. A protected band is detected in MBH-POA and AMY mRNA (indicated by the arrow) indistinguishable from that observed using samples of RNA prepared from R2C cells and rat ovary

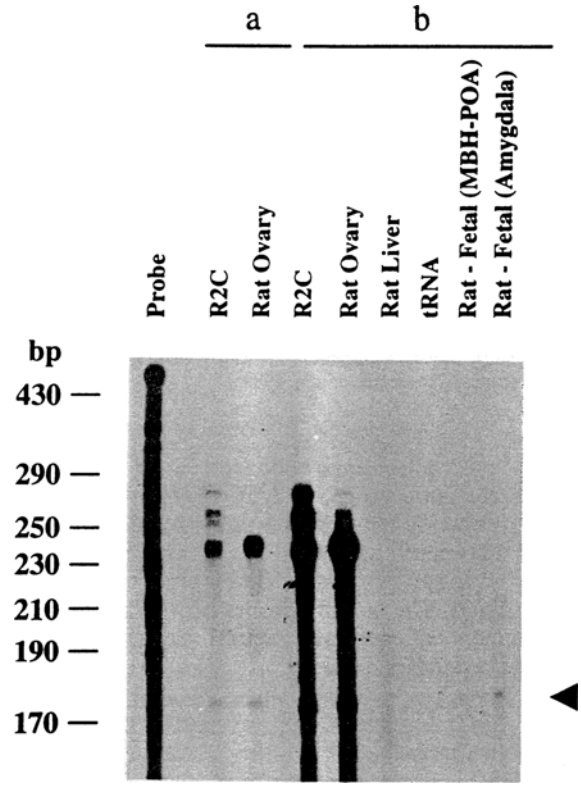

Figure 6 S_1 Nuclease protection analysis of total RNA from a rat Leydig tumor cell line (R2C), rat ovary, rat liver, and rat fetal brain [medial basal hypothalamic and preoptic area (MBH-POA) and amygdala (AMY)] using a probe that spans the promoter utilized in rat ovary and R2C cells. The RNA samples (10 μg) were hybridized to a uniformly labeled probe spanning the transcription initiation sites used in rat ovary and R2C cells (− 144 to + 244; as shown in Figure 2B). The position of the size markers are shown to the left (based upon a sequencing ladder that was utilized as reference which is not shown in this exposure). (**a**) Indicates a short exposure (2 days) of the R2C and rat ovary mRNA samples. (**b**) Indicates a long exposure (5 days) of all mRNA samples. The lane containing tRNA served as negative control. A partially protected band was detectable in MBH-POA and AMY mRNA (indicated by the arrow). This same fragment was observed as a minor band in mRNA samples from R2C cells and rat ovary. Note that the experiments depicted in Figures 5 and 6 were performed in parallel and employed identical methods for the preparation of the probes

rats older than gestational day 20 contains a 5′ terminus different than that derived from the 'ovarian' aromatase promoter. Indeed, the partially protected band observed in brain samples using the 'ovarian probe' would correspond to the use of a 3′ splice acceptor site immediately 5′ to the aromatase open-reading frame, but 3′ to the 'ovarian' promoter.

This inference has been confirmed by the isolation and characterization of the 5′ termini of aromatase mRNAs from perinatal rat amygdala and MBH-POA. The sequences are identical 5′ to an AG dinucleotide located 40 nucleotides 5′ to the initiator methionine, indicating that at this stage of development, the expression of aromatase in both of these neural structures is controlled by a distinctive promoter. As expression of aromatase in brain is a consistent feature in species as diverse as birds to man (Naftolin *et al.*, 1975; Callard *et al.*, 1978a,b), it is tempting to speculate that regulation of $P\text{-}450_{AROM}$ in neural tissue in other species may also involve similar, if not identical, mechanisms. The sequence of the MBH-POA/amygdala aromatase 5′ terminus shows no high degrees of homology to sequences contained in the GenBank database. The significance of distant similarities to other rat neural mRNAs and viral sequences is not obvious.

These studies demonstrate that at least two mechanisms exist in rat tissues and cell lines to effect differences in the expression of aromatase. In the rat ovary and in the Leydig cell tumor line R2C, different patterns of aromatase are controlled via influences exerted through promoter elements located immediately 5′ to the aromatase open-reading frame. By contrast, we demonstrate for the first time that $P\text{-}450_{AROM}$ mRNA transcripts present in neural tissue during perinatal development contain a distinctive 5′ terminus and are derived from a different promoter compared to that which controls aromatase expression in R2C and rat ovary.

Despite these insights, important questions remain. It is not known whether the genomic elements that control $P\text{-}450_{AROM}$ expression in neural tissue remain the same during all stages of development. This is

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Amy,MBH-POA GTG ACC AGC AGA TCC AGA ACT TTA CAT TTT TCC TTC TGA GGC AAA ATA
               **           *   *** *** *** *** *** *** *** *** *** *** ***
Ovary,R2C   CTG CTT TCT TCC TAT AGA ACT TTA CAT TTT TCC TTC TGA GGC AAA ATA
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Figure 7 Nucleotide sequence of primer-extended clones isolated from late gestation age rat hypothalamic and preoptic area (MBH-POA) and amygdala (AMY) RNA compared to the 5′ termini determined for aromatase mRNA expressed in rat ovary and rat R2C cells. The boxed AG dinucleotide (nucleotides + 57–58) represents the site of divergence in the aromatase mRNA in RNA prepared from late gestational rat MBH-POA and AMY tissue from the nucleotide sequence of the 5′-flanking segment of the rat aromatase gene. This corresponds to potential splice acceptor site 'a' in Figure 1. The underlined AG dinucleotide (nucleotides + 80–81) corresponds to the acceptor splice site utilized in extragonadal tissues of the chicken (9) (shown as the boxed AG dinucleotide 'b' in Figure 1). Only distant similarities of the AMY and MBH-POA aromatase 5′ terminus sequence to that of other rat mRNAs or viral sequences (e.g., rat Na^+/K^+ ATPase alpha-2 subunit, rat glutamate receptor and *E. coli* ribonucleoside diphosphate reductase) were identified when compared to the GenBank nucleotide sequence database using the GCG WordSearch program (Devereaux *et al.*, 1984)

particularly intriguing in light of the fact that P-450_{AROM} expression is differentially regulated by androgens between the MBH-POA and AMY brain regions during postnatal development (Roselli & Resko, 1993). The specific probes now available for the measurement of perinatal neural aromatase mRNA make such studies feasible.

Materials and methods

RNA preparation

RNA was prepared by homogenization of tissue specimens or R2C cell pellets in guanidium isothiocyanate followed by centrifugation over a 5.7 M cushion of cesium chloride (Chirgwin *et al.*, 1979). RNA was prepared from random cycling adult rat ovary and liver (– 250 grams). Samples of ovary and placenta from pregnant rats were obtained at days 19 to 20 of gestation. Tissue from the hypothalamus and/or amygdala was dissected from fetal rats on gestational days 19 to 20. Polyadenylated RNA was prepared using oligo dT cellulose chromatography (Aviv & Leder, 1972). Due to the time span during which these experiments were performed, several different preparations of ovary and R2C RNA were employed. For this reason, the precise levels of aromatase mRNA detected can be compared only in Figures 5 and 6, which employed the same preparations of mRNA.

Genomic clone isolation and characterization

A rat genomic DNA library (Clontech Laboratories, Inc., Palo Alto, CA) was screened with a 261 bp probe (derived from the EcoRI–NcoI restriction endonuclease sites) of the 5′ region of the rat cDNA that was labeled by random hexamer priming using [α-^{32}P]dCTP (Feinberg & Vogelstein, 1983). Three recombinant clones were isolated and purified. One clone was characterized in detail and found to contain an insert of genomic DNA approximately 15 kb in length. Southern analysis localized the segment flanking the 5′ terminus of the aromatase open-reading frame to a 1.5 kb PstI restriction endonuclease fragment (see Figure 2A) which was subcloned into Bluescript (BS) KS M13+ vector (Stratagene, San Diego, CA). This plasmid was designated BSrAromPstI.

Hybridization probes and S_1 nuclease protection

Two uniformly labeled probes were utilized in these studies. The first probe was a 388 bp fragment containing a portion of exon 1 and the 5′ flanking region of the rat P-450_{AROM} gene (see Figure 2B). To isolate this segment, two oligonucleotide primers were synthesized (Applied Biosystems, Foster City, CA); RP1s: 5′-ACAAGCTTACGAATTCGCAAGATGATAAGGTTCTATCAGACCAAC-3′; the underlined nucleotide (*nt*) corresponds to the base underlined in the oligonucleotide sequence and this designation is used throughout the text; *nt* = – 130, see Figure 1; RP2as: 5′-ACAAGCTTACCTGGTATGGAAGATGAGCTCTCAC-3′; *nt* = 210 (Lephart *et al.*, 1990). These oligonucleotides contain artificial HindIII restriction sites at their termini. The fragment bounded by these oligonucleotides was amplified for 35 cycles, digested with HindIII, and the products were electrophoresed on a 2% low melting temperature agarose gel. After purification, the 388 nucleotide fragment was ligated into HindIII digested Bluescript (BS)KSM13+ vector to yield that plasmid BSrAromPromI.

A second probe complementary to a segment of the aromatase mRNA coding region was prepared [using the SacI–BamHI restriction endonuclease sites (*nt* 208 and 692, respectively) (Lephart *et al.*, 1990)] by ligating this 484 nucleotide fragment into digested Bluescript (BS) KSM13+ vector to yield the BSrArom4845 plasmid.

S_1 nuclease protection assays were performed using uniformly labeled RNA probes according to the method of Burke (1984). A probe spanning the region immmediately 5′ to the initiator methionine was prepared by digestion of BSrAromPromI with EcoRI. After linearization, the probe was synthesized using T_3 RNA polymerase to transcribe a uniformly labelled probe with a polarity complementary to that of aromatase mRNA. In like fashion the second probe was synthesized by linearization of BSrArom4845 with the restriction endonuclease NcoI and followed by incubation with T7 RNA polymerase to yield a probe 430 nt long that is complementary to the coding sequence of the aromatase mRNA [nucleotides 262 to 692 (Lephart *et al.*, 1990)]. Importantly, in experiments in which the abundance of aromatase mRNA was quantitated using two different probes, the probes were synthesized in parallel using concentrations of [^{32}P]UTP (800 Ci/mMol) to yield probes of similar specific activity ($\sim 10^9$) d.p.m./μg) (see Figure 2D).

Primer-extension analysis

Primer extension was performed using the protocol of Calzone *et al.*, (1987). Ten micrograms of total RNA from rat ovary, R2C, rat ovary during pregnancy, fetal rat brain [i.e., medial basal hypothalamic and preoptic area (MBH-POA) (4)] and 5 μg poly(A)$^+$ RNA from R2C were hybridized to an end-labeled primer [1×10^7 c.p.m.; oligo PEXT Rat AROM 5′-ACAAGCTTACCTGGTATGGAAGATGAGCTCTCAG-3′, *nt* = 210 (Lephart *et al.*, 1990) shown as C in Figure 2] at 60°C for 1 h. Primer extension was performed using avian myelobastosis virus reverse transcriptase (Seikagaku-America, Rockville, MD) at 42°C for 1 h. The products of the reaction were precipitated with ethanol, electrophoresed on a 6% denaturing acrylamide gel, dried and visualized by autoradiography.

cDNA library construction and screening

Ten micrograms of polyadenylated mRNA prepared from the R2C cell line was annealed to the oligonucleotide primer [5'-TATAATGTCACCATCATGGTCCCGG-3', *nt* = + 120 (Lephart *et al.*, 1990)] for 1 h at 60°C. After extension, second strand synthesis was accomplished according to the method of Gubler and Hoffman (1983). Synthetic EcoRI adapters were attached and the cDNA that was then ligated into phosphatased λgt10 arms (Promega, Madison, WI), packed *in vitro* and plated onto bacteria. The library was then screened with a sense oligonucleotide (5'-TCCTTCTG-AGGCAAAATACCACAAGATG-3', *nt* = + 88, see Figure 1) yielding one positive clone.

Preparation and screening of RACE primer-extended library

For brain samples, 10–20 μg of total RNA prepared from MBH-POA or amygdala (AMY) tissue sites were processed using a 5'-AmpliFinder RACE Kit (Clontech Laboratories, Palo Alto, CA). First strand cDNA synthesis was primed using a oligonucleotide corresponding to nucleotides [5'-CAC-AGCAACTACATCTCCAGATTCG-3', *nt* = 403 (Lephart *et al.*, 1990)]. Reverse transcription, RNA hydrolysis, cDNA purification, and anchor ligation were performed according to the protocols provided by the manufacturer. Amplification of the products of these reactions utilized two primers: one corresponding to the anchor primer and a second oligonucleotide located 5' to that used in the first strand cDNA synthesis [5'-CTCATCATCAGCAAGTCCTTGAGCATGG-TC-3', *nt* = 380 (Lephart *et al.*, 1990)]. The cDNA was digested with EcoRI, ligated, packaged, and plated onto bacteria. The libraries were screened with a third oligonucleotide that corresponds to a sequence located 5' to those used to prime first strand cDNA synthesis and PCR amplification [5'-TGTCTGGGAATCGGGCCTCTCATTT-3', *nt* = 235 (Lephart *et al.*, 1990)].

DNA sequence analysis

Restriction endonuclease fragments were ligated into the plasmid vector Bluescript or into M13 single-stranded phage vectors and sequenced using Sanger dideoxy sequencing protocols (Sanger *et al.*, 1977).

Computer analyses of nucleotide sequences

A comparison of the nucleotide sequence of the 5' terminus of the AMY and MBH-POA p-450$_{AROM}$ cDNA to the GenBank nucleotide sequence bank was made using the genetics computer sequence analysis software program WordSearch from the University of Wisconsin Genetics Computer Group (Devereaux *et al.*, 1984).

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