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Modulation of Preoptic Regulatory Factor-2 (porf-2) mRNAs by Castration and Hypophysectomy

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Neuropeptides are central to the regulation of mammalian gender-dependent development and reproduction. Preoptic regulatory factor-2 is a neuropeptide gene that is known to be expressed in rat brain and testis *(1)*. In the brain, expression is gender-dependent and age-dependent *(2,3)*. Tissue-specific transcripts are found in the preoptic area (POA) of the hypothalamus and in the testis *(1)*. In order to investigate the effects of reproductive hormone status on expression of porf-2 in the male rat, porf-2 transcripts were studied by Northern blot analysis in intact, hypophysectomized, and castrated rat POA, medial basal hypothalamus (MBH), cerebral cortex (CC), testis, and liver. Castration or hypophysectomy increased levels of the brain-specific 0.84 kb 5' porf-2 transcript in the POA, but did not affect levels of this transcript in the CC. There was a small decrease in the MBH following castration. Hypophysectomy also resulted in a fourfold increase in the 5' 1.1 kb testis-specific transcript. The affected transcripts are localized to the cytoplasm. A nontissue specific 3' transcript was also detected. Interestingly, this 0.6 kb transcript became nondetectable in all tissues examined following hypophysectomy. Porf-2 mRNA was also detected in human hypothalamus, testis, adrenal, placenta, and prostate with unique transcripts in each tissue examined. It has been shown elsewhere that porf-2 is a unique single copy gene in the rat genome. These data demonstrate that expression of the porf-2 gene is differentially regulated at the pretranslational level by intrinsic tissue-specific, as well as extrinsic pituitary and gonadal factors. The selected responses to reproductive hormonal status suggest that porf-2 may play a role in hypothalamic pituitary–gonadal interactions.

Key Words: Neuropeptide; gene regulation; castration; hypophysectomy; reproduction; hypothalamus; preoptic regulatory factors.

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Introduction

Neuropeptides are central to the regulation of mammalian gender-dependent development and reproduction. The preoptic regulatory factor-2 gene is a unique neuropeptide gene that is expressed in rat brain and testis *(1)*. Brain expression is regionally controlled in an age- and gender-dependent manner *(2,3)* leading to the hypothesis that porf-2 has a role in gender-dependent development and function. During postnatal development, porf-2 mRNA shows a female specific rise in level of expression in the hippocampus between 15 and 60 d of age. No change in expression is seen in male rat hippocampus during the same time period. In contrast, porf-2 mRNA in cerebral cortex (CC) and medial basal hypothalamus (MBH) declines in both male and female rats during this pre- to peri- to postpubertal transition *(3)*. In the aging rat, porf-2 mRNA levels remain stable in the CC and MBH between 2 and 24 mo of age, but begin to decline in the preoptic area (POA) as early as 6 mo of age. In the hippocampus, porf-2 expression shows a peak of activity at 6 and 12 mo when compared with both younger (2 mo) and older (24 mo) male rats.

A 75 amino acid peptide with a hydrophobic secretory leader can be translated in vitro from the porf-2 brain RNA. It was hypothesized that if this porf-2 peptide mediates gender-related brain function, then the expression of the porf-2 gene would be modified by testicular and pituitary factors. It was also reasoned that if the porf-2 gene has an essential role in mammalian development or reproduction it would be transcribed in more than one mammalian species.

In this report the previous analyses of rat porf-2 transcripts *(1)* were extended to include the MBH, the CC, and the liver. It was assessed whether the pretranslational expression of the porf-2 gene is regulated by pituitary and testicular factors in a tissue-dependent manner, by Northern blot analysis of RNA from hypophysectomized and castrated male rats. It was determined if the regulated brain and testis-specific transcripts are in the cytoplasm and thus available for translation. Finally, the author examined selected human steroid-responsive endocrine organs, including the hypothalamus, adrenal, placenta, testis, and prostate, for interspecies conservation of porf-2 transcripts.

Materials and Methods

Reagents

All culture media components were supplied by Difco (Detroit, MI). Competent DH5α cells, yeast tRNA, and RNA molecular weight gel standards were obtained from Bethesda Research Labs (BRL; Gaithersburg, MD). Sequencing primers, deoxynucleotides, dideoxynucleotides, SP6 and T7 polymerases, ribonuclease inhibitor, RQ1 DNase, pGEM3Z, and pGEM4Z plasmids and ribonucleotides were purchased from Promega (Madison, WI). Restriction endonucleases were purchased from BRL, Promega, New England Biolabs (Beverly, MA), or IBI (New Haven, CT). ^{32}P-UTP and ^{35}S-dATP were obtained from Amersham (Arlington Heights, IL). Molecular biology grade agarose and phenol were from IBI. Urea (Ultrapure) came from Schwartz-Mann (Cleveland, OH). Sodium dodecyl sulfate (SDS) and formamide (purissima) were from Fluka (Uppsala, Sweden). Dithiothreitol and ATP came from Pharmacia (Piscataway, NJ); the Pentex bovine albumin, Fraction V, from Miles Laboratories (Kankakee, IL); Type 7 oligo (dT) cellulose from Collaborative Research (Milwaukee, WI); nuclease free bovine serum albumin (BSA), T4 DNA ligase, calf intestinal alkaline phosphatase and DNA polymerase I (Klenow fragment) from Boehringer Mannheim (Indianapolis, IN). Guanidine hydrochloride, chloroform, absolute ethanol, sodium chloride, and sodium citrate were from Mallinkrodt (Paris, KY). All other chemicals were obtained from Sigma (St. Louis, MO).

Animals

Male Sprague-Dawley CD rats were obtained from Charles River Laboratories (Wilmington, MA) and housed under conditions of 14 h light, 10 h dark with food and water *ad libitum*. All animal handling was done according to institutional guidelines, which are AAALAC approved. Rats were sacrificed at 51–55 d of age either intact, 10 d postcastration, or 10 d after hypophysectomy. Castration and hypophysectomy were verified at the time of sacrifice. Each sample used in the hormonal studies represents an aliquot of RNA pooled from 19–20 rats. The cytoplasmic RNA samples were aliquots of tissues pooled from 6–10 rats. The MBH was dissected by removing a 1.5 mm deep section 1 mm lateral to the midline, anterior–posterior from the optic chiasm to the mammillary bodies (average wet weight 34 mg). This region includes the median eminence and arcuate nucleus. The POA was dissected as a 3 mm wide, 2 mm anterior–posterior, and 1.5 mm deep block at midline just anterior to the MBH, using the anterior commissure as the rostral landmark. The average wet weight was 42 mg. The medial preoptic and anterior hypothalamic nuclei are included in this sample. The CC and liver were sampled randomly. Testes were decapsulated before freezing a representative sample. All tissues were removed between 1300 and 1600 h, weighed and frozen in liquid nitrogen within 3 min of death. All tissue samples within a treatment group were from the same animal pool.

Human Tissues

The human tissues were obtained at surgery, autopsy, or delivery with approval of the Institutional Review Board, and rapidly dissected into small pieces. Adrenal and placenta were frozen in liquid nitrogen; hypothalamus, testis, and prostate were placed on dry ice, then stored at –80°C before extraction. The hypothalamus, testis, and placenta were normal specimens obtained at autopsy, orchiectomy, and delivery, respectively. The adrenal tissue was from the contralateral gland in a case of hyperplasia. The prostate tissue was from a subject with benign prostatic hypertrophy. The adrenal, hypothalamus, testis, and prostate were all from adult subjects.

RNA Preparation

Total tissue RNA was extracted, oligo (dT) purified twice with dimethylsulfoxide dispersion and analyzed for integrity by in vitro translation as previously described *(1)*. Cytoplasmic RNA was isolated from the postnuclear supernatant following removal of nuclei by centrifugation through a sucrose cushion *(4)*. All RNA samples were quantified and assessed for purity by UV spectrophotometry at 260 and 280 nm.

Riboprobe Preparation

Restriction fragments derived from rat porf-2 cDNA clones (Fig. 1) were subcloned into the pGEM3Z and pGEM4Z in vitro transcription vectors, and sequence analysis was done by the double stranded dideoxynucleotide chain termination technique *(5,6)* using ^{35}S-dATP. Strand-specific RNA probes (riboprobes), labeled to a specific activity of 0.5–1 × 10^9 cpm/μg with α-^{32}P-UTP, were generated by in vitro transcription using T7 or SP6 polymerase according to the protocol provided by Promega. Templates were digested with RQ1 DNase. Probe A is a 375 nt cRNA derived from the 5' *Eco*RI-*Sph*I restriction fragment subclone. Probe Y is a 644 nt cRNA transcribed from the 3' *Hin*cII-*Eco*RI restriction fragment subclone.

Northern Blot Hybridization Analysis

Polyadenylated RNA (3 to 5 μg) was electrophoresed through 1.4% agarose formaldehyde gels, then transferred *(7)* and UV crosslinked *(8)* to nylon filters (MSI, Westborough, MA). Prehybridization was performed in 500 m*M* sodium phosphate, pH 7.2, 0.1% nuclease free BSA, 1 m*M* ethylenediamine-tetra-acetic acid, 7% SDS at 65°C for 2–3 h. Fresh solution containing 0.5–1 × 10^7 cpm/mL probe was added and hybridization continued for 16–20 h. Filters were washed according to the protocol of Church and Gilbert with a final wash in 100 m*M* sodium phosphate, pH 7.2, at 65°C for the human RNA blots and at 72°C to 80°C for the rat RNA blots. The filters were washed until no signal was detected by scanning with a Geiger monitor (1.5–3.0 h).

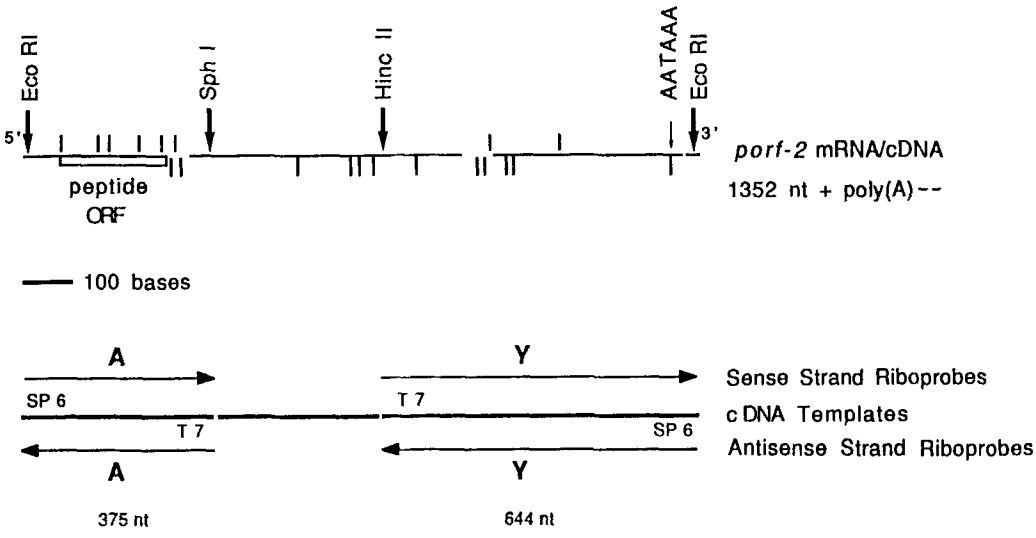

Fig. 1. Diagram of the porf-2 POA mRNA and corresponding region-specific DNA templates and riboprobes. *Eco*RI, *Sph*I, and *Hin*cII restriction enzyme cleavage sites and the polyadenylation site, AATAAA, are indicated by the vertical arrows. Restriction enzyme fragments of the full length porf-2 cDNA were subcloned into dual promoter pGEM transcription vectors. SP6 and T7 refer to the respective RNA polymerases used to synthesize the RNA probes A and Y as indicated by the horizontal arrows. The probes and mRNA are drawn to the same scale. Vertical lines above and below the mRNA show the locations of start and stop signals. The 5' porf-2 peptide open reading frame is depicted by the unshaded rectangle. Probe template sequences were verified using the dideoxy chain termination method as described in Materials and Methods.

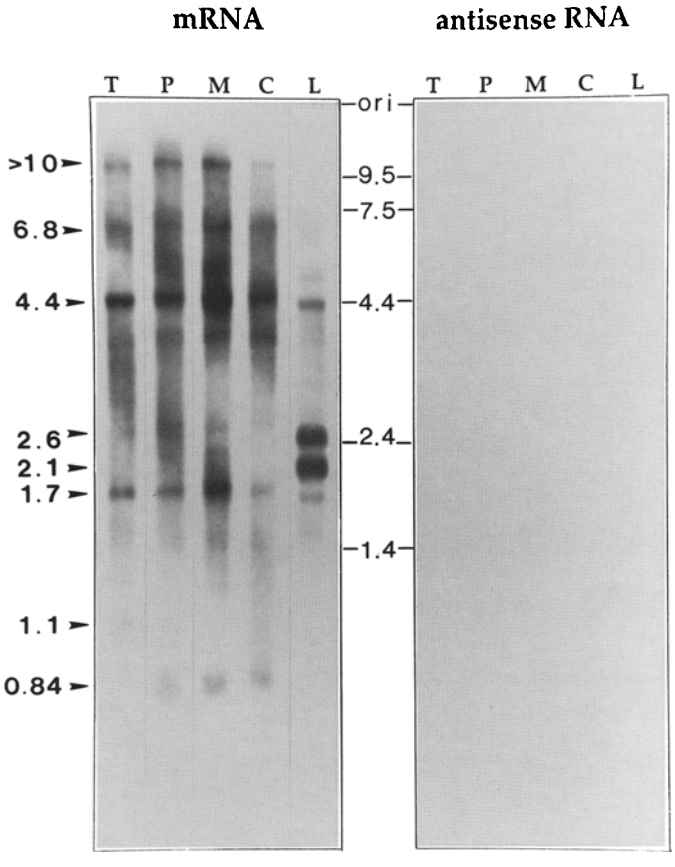

Fig. 2. Northern blot analysis of porf-2 messenger and antisense RNA in rat brain, testis, and liver. Three micrograms oligo (dT) selected RNA from intact male rats was isolated, quantified, electrophoresed, and hybridized as described in Materials and Methods. The left panel shows the result of hybridization using ^{32}P-cRNA complementary to the 5' end of porf-2 mRNA. The panel on the right shows the absence of hybridization when the colinear synthetic ^{32}P-mRNA is used as the hybridization probe. P, preoptic anterior hypothalamus; M, medial basal hypothalamus; C, cerebral cortex; T, testis; L, liver. Transcript molecular size estimates shown in kilobases on the left are based on the migration positions of ethidium bromide stained 0.24–9.5 kb RNA standards shown in the center.

Autoradiography was done for 2.5–22 h. Molecular size estimates shown in kilobases are based on ethidium bromide stained 0.24–9.5 kb RNA standards.

The densitometry results are based on duplicate Northern blot analyses of pooled RNA samples obtained from 19 or 20 rats in each group. An LKB Ultroscan linear densitometer was used to quantify autoradiographic signal intensity. Background was determined separately for each lane as the average of the 16 lowest measurements. Values are expressed in absorbance units and are the average of two determinations. Autoradiographic exposures were adjusted so that the intact samples were within 10%. Reported duplicate values fell within a ±20% range.

Results

Tissue Distribution of porf-2 RNA Transcripts in the Intact Male Rat

Porf-2 mRNA is found in the POA and testis as previously described *(1)*, using the 375 nt 5' probe A complimentary to the message strand. As shown in Fig. 2 (left panel) porf-2 mRNA is also detected in the MBH, CC, and liver. The previously described *(1)* tissue-specific 1.1 kb testis transcript and 0.84 kb POA transcripts are also seen. In addition, the

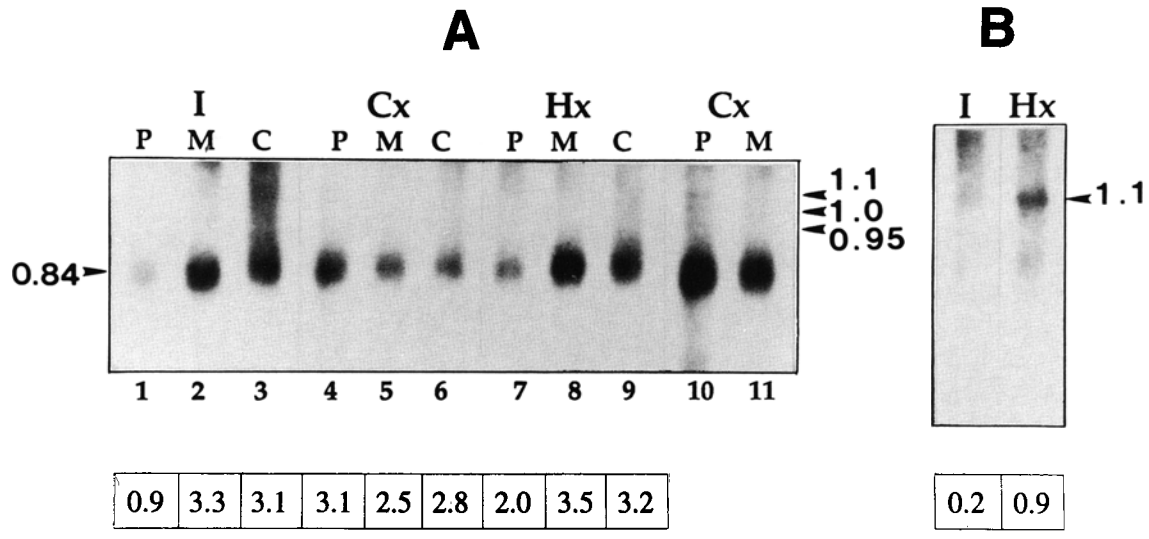

Fig. 3. Effects of castration and hypophysectomy on brain **(A)** and testis-specific **(B)** porf-2 transcripts. Three micrograms polyadenylated RNA isolated from intact (I), castrated (Cx) or hypophysectomized (Hx) male rats was analyzed by Northern blot hybridization as described in Materials and Methods, using the 375 nt 5' cRNA probe A. P, preoptic anterior hypothalamus; M, medial basal hypothalamus; C, cerebral cortex. Lanes 10 and 11 have been overexposed to show the 1.1, 1.0, and 0.95 kb transcripts in the POA of the Cx rat. Estimated sizes of the affected transcripts are indicated in kilobases. The average densitometry readings for the 0.84 kb brain transcripts and the 1.1 kb testis transcripts are shown in the boxes below (n = 2 samples from pools of 20–24 rats).

0.84 kb transcript is found in the other brain regions examined, the MBH and CC. No RNA is detected when the synthetic mRNA strand is used as the probe (right panel).

Effects of Hypophysectomy and Castration on Specific porf-2 Transcripts

In order to investigate the effects of pituitary and testicular factors on rat testis and brain porf-2 transcripts, Northern blots of RNA extracted from intact, castrated, and hypophysectomized male rats were compared.

Castration and Hypophysectomy Increase the 0.84 Kb Brain-Specific Transcript

In response to castration, levels of the brain-specific 0.84 kb porf-2 transcript are altered in a region-specific manner. The levels increase threefold in the POA (3.1 vs 0.9 AU, lanes 1 and 4, Fig. 3A), showing no change in MBH and CC. Following hypophysectomy, there is a twofold increase in this transcript in the POA, but no change in either MBH or CC. Castration also results in the appearance of three porf-2 RNA species not previously detected in the rat brain. These 0.9, 1.0, and 1.1 kb transcripts (lane 10) are seen only in the POA. The other porf-2 transcripts that are detected in the brain with the 5' probe A are not affected by castration.

Hypophysectomy Affects the 1.1 Kb Testis-Specific Transcript

The testis-specific 1.1 kb porf-2 transcript shows a fourfold increase following hypophysectomy (0.9 vs 0.2 AU, Fig. 3B). There is no effect on the 1.7 kb transcript that is seen in all tissues (4.6 vs 4.2 AU) or on the other nontissue-specific transcripts, following hypophysectomy.

Both Castration and Hypophysectomy Affect a 3' 0.6 Kb Transcript

A Northern blot hybridization analysis with the 3' 644 nt probe Y (Fig. 1) reveals a 600 nt band not detected with the 5' probe (Fig. 4). This band is found in brain and liver and, to a lesser degree, testis. Castration results in a modest decrease in intensity of this band in MBH (22%) and CC (31%), and hypophysectomy causes this RNA species to fall below the limits of detection for this assay in each tissue examined. The 10, 6.8, 4.4, and 1.7 kb nonspecific as well as the 2.6 and 2.1 kb liver-specific bands plus the 1.1 kb testis-specific band and the 0.84 kb brain-specific bands are also detected with this 3' probe (data not shown).

Confirmation that Hormone-Responsive, Tissue-Specific porf-2 Transcripts Are Localized in the Cytoplasm

Northern blot analysis was repeated using cytoplasmic polyadenylated RNA from brain and testis using the 5' probe as shown in Fig. 5. The 0.84 kb brain-specific and 1.1 kb testis-specific transcripts, as well as the 1.7 and 4.4 kb ubiquitous transcripts, were detected in the postnuclear supernatant.

Effects of Castration and Hypophysectomy on Yields of Total and Polyadenylated RNA

The yields of total and polyadenylated RNA from liver, testis, CC, MBH and POA from intact, castrated, and hypophysectomized rats are shown in Table 1. When submitted to linear regression analysis, no positive or negative correlation was found between the yield of total or polyadenylated RNA and the densitometry readings for the specific 0.84 or 0.6 kb transcripts.

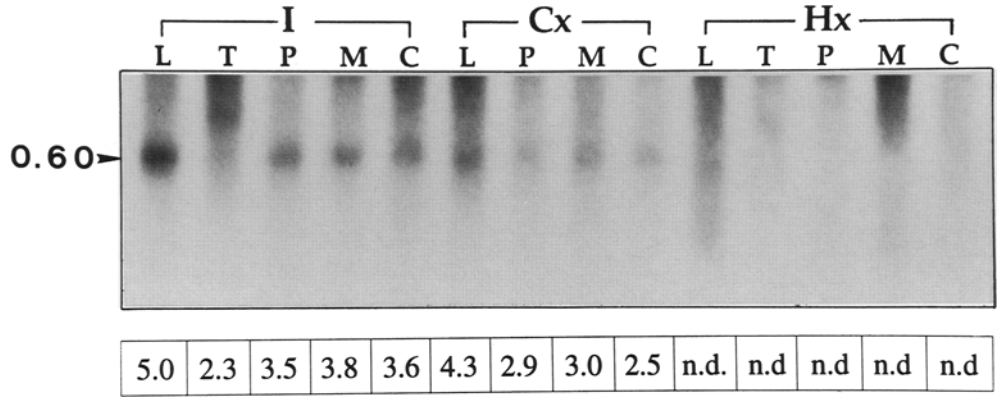

Fig. 4. Effect of hypophysectomy on a 3' porf-2 RNA. Three micrograms polyadenylated RNA was isolated, quantified, electrophoresed, and hybridized as described in Materials and Methods using the 644 nt 3' cRNA probe Y. I, intact; Cx, castrated; Hx, hypophysectomized male rats; L, liver; T, testis; P, preoptic anterior hypothalamus; M, medial basal hypothalamus; C, cerebral cortex. The 0.6 kb transcript indicated is detected only with the 3' porf-2 probe Y. The average densitometry readings for the 0.6 kb transcripts are shown in the boxes below (n = 2 samples from pools of 20–24 rats).

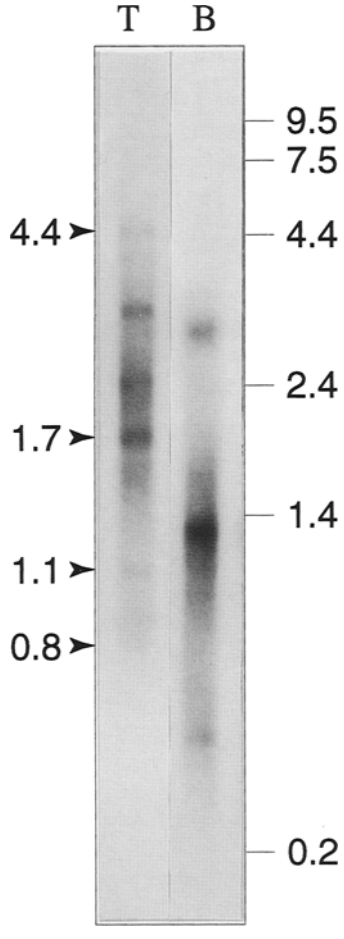

Fig. 5. Detection of tissue-specific porf-2 transcripts in postnuclear supernatant. Three micrograms postnuclear RNA was oligo (dT) selected, quantified, and electrophoresed as described in Materials and Methods. Northern blot hybridization was done using the 5' 375 nt porf-2 probe A. T, testis; B, brain. Transcript sizes are indicated in kilobases on the left. Molecular weights on the right show migration positions of ethidium bromide stained 0.24–9.5 kb RNA standards.

Table 1
Yield of Total and Polyadenylated RNAs[a]

Tissue		Total RNA[b] mg per g tissue	PolyA$^+$ RNA[b] mg per g tissue
Liver	N	6.1	31.6
	C	6.9	28.4
	H	6.5	24.9
Testis	N	2.4	79.5
	H	1.3	61.7
Cortex	N	1.0	15.7
	C	0.8	16.1
	H	1.1	16.2
MBH	N	1.2	22.6
	C	1.0	17.7
	H	1.2	20.2
POA	N	1.0	26.6
	C	1.1	24.7
	H	0.9	21.0

[a]Abbreviations: C, castrated; H, hypophysectomized; N, normal.
[b]Values in the last two columns represent the average of two determinations. No significant correlation was found between the total or relative yield of polyA RNA and specific RNAs as measured by densitometry.

Porf-2 mRNAs Are Found in Human Tissues

Human RNA from hypothalamus, adrenal, placenta, prostate, and testes was analyzed by Northern blot hybridization using the 5' porf-2 rat cRNA probe. Very low amounts of a 12 kb transcript are detected in all tissues studied (not shown). In addition, as shown in Fig. 6, tissue-specific porf-2 transcripts are detected. These include two hypothalamic transcripts of 3.4 and 1.9 kb, a 1.5 kb transcript in adrenal and placenta, a 1.2 and a 0.9 kb transcript unique to placenta, and a 5.4 kb testis transcript.

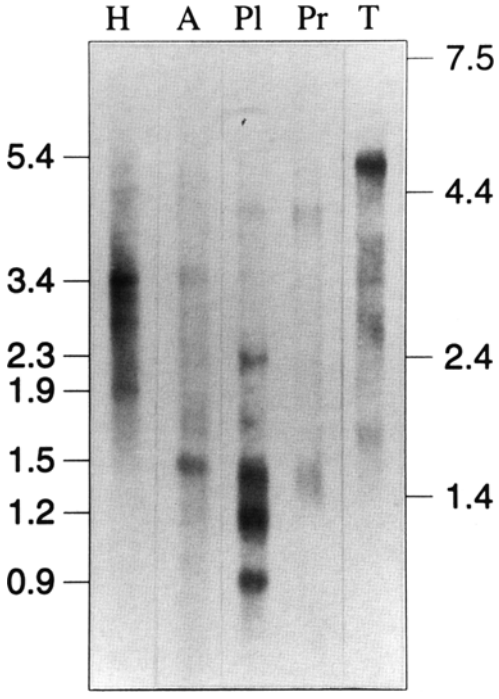

Fig. 6. Detection of porf-2 related transcripts in human hypothalamus, adrenal, placenta, prostate, and testis. Each lane contains 4 or 5 μg oligo (dT) selected RNA isolated, quantified, electrophoresed, and hybridized as described in Materials and Methods using the rat 5' 375 nt porf-2 cRNA probe. H, hypothalamus (5 μg); A, adrenal (4 μg); Pl, placenta (4 μg); P, prostate (4 μg); T, testis (4 μg). Transcript sizes are indicated in kilobases on the left. Molecular weights on the right show migration positions of ethidium bromide stained 0.24–9.5 kb RNA standards.

Discussion

Several porf-2 transcripts are seen in each tissue examined. A unique single copy porf-2 gene is present in the rat genome (JG Golden and FV Nowak, unpublished observation). Therefore, all of the observed transcripts are derived from a single DNA locus. Unique, tissue-specific porf-2 RNAs are also seen. The finding of multiple and tissue-specific transcripts, therefore, suggests alternative RNA transcription, splicing, or processing in response to intrinsic tissue or cell-type specific signals. Tissue-specific processing of polypeptide RNAs has been well described *(9)*. Specific examples include calcitonin gene-related peptide (CGRP) *(10)* and the opioids *(11,12)*. The presence of tissue-specific transcripts has been shown to have functional consequences by altering the nature of the translated protein *(10,13)*, translational efficiency *(14–16)*, the stability and functional half-life of the RNAs *(17)*, or transcriptional regulation *(18)*. The tissue-specific brain and testis transcripts are found in the cytoplasm and thus are available for translation.

Both gonadectomy and hypophysectomy affect porf-2 transcripts. Specific changes in selected tissue-specific transcripts in response to hormonal status implies that factors from the pituitary and testis either directly or indirectly influence porf-2 mRNA transcription, processing, or stability.

Both steroid and polypeptide hormones are known to directly influence gene transcription and processing *(19–24)*. The enhancement of the 0.84 kb porf-2 RNA in the POA by castration suggests that testicular factors have a direct or indirect repressive effect on this porf-2 RNA in the intact male rat in this brain region. Interestingly, this effect is not seen in the MBH and CC. This suggests that intrinsic region-specific porf-2 RNA transcription or stability factors interact with or respond differentially to gonadal status in different regions of the rat brain. The observation that hypophysectomy results in a similar effect suggests that this is a direct effect of gonadal factors rather than an indirect one via pituitary hormones. However, this remains to be established.

Coexistence of positive and negative regulatory elements to the same hormone, thyroid hormone, has been described for the growth hormone gene *(25)*. In addition, it is now well-established that many hormones regulate transcription through complex hormone response units (HRUs). HRUs include various combinations of accessory factors and coregulators that may activate or repress transcription through interaction with the basal transcription complex. Differential tissue or cell-type distribution of accessory factors and coregulators could explain the regional difference in porf-2 response.

Finally, it has now been demonstrated that porf-2 hybridizing transcripts are found in human tissues, including hypothalamus and testis. The existence of related transcripts in a second species indicates that porf-2 sequences have been evolutionarily conserved and argues for an essential function of the porf-2 gene. The rat and human testicular transcript sizes differ. However, interspecies variations in transcript length and splice sites are common *(13,26)*.

In summary, these data support a specific hormone responsive role of porf-2 gene products in the hypothalamus and testis. Further studies are required to delineate the functional consequences of the observed alterations in porf-2 transcripts and the mechanism whereby pituitary and testicular factors alter their expression in testes and brain, respectively.

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