Poly(A) tail length of oxytocin- and lysine vasopressin-encoding mRNAs increases during development in the porcine hypothalamus

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ABSTRACT

Lysine vasopressin- and oxytocin-encoding mRNAs have been analysed in the developing hypothalamus of the pig. The two hormone-encoding mRNAs were first detectable on fetal day 49 by Northern blot analysis. Whereas RNase mapping revealed identical transcripts throughout the developmental stages studied, Northern blots showed that the early transcripts appeared to be shorter (by 100–200 nucleotides) and more heterogeneous in size than those of later stages. This developmentally related length polymorphism was shown to be due to different poly(A) lengths and was abolished by removal of the poly(A) tails with RNase H. These results indicate that maturation of neurones in the developing porcine hypothalamus is accompanied by an increase in length of the poly(A) tail of vasopressin and oxytocin mRNAs.

Journal of Molecular Endocrinology (1990) 4, 151–158

INTRODUCTION

The mammalian nonapeptide hormones vasopressin and oxytocin are synthesized from precursor molecules encoded by closely related genes (Richter, 1985; Ivell, 1986). The two genes are expressed in distinct populations of highly differentiated neurosecretory cells: the magnocellular neurones of the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus (Mohr, Bahnsen, Kiessling & Richter, 1988). In the rat, the most thoroughly studied system to date, the expression of the vasopressin and oxytocin genes is an early event in the differentiation of these cells. Neurophysin-immunoreactive material is already present at fetal day (FD) 16, before the first neurosecretory neurones can be identified by their ultrastructural features (Castel, Gainer & Dellmann, 1984). Using in-situ hybridization techniques, vasopressin-encoding mRNA is detectable around FD 15–16, and oxytocin-specific transcripts between FD 17 and 19 (Fehr, Schmale & Richter, 1989; Laurant, Hinde¬lang, Klein et al. 1989). Accumulation of the vasopressin and oxytocin transcripts increases throughout subsequent pre- and postnatal development, reaching adult levels around postnatal day 30 (Almazan, Lefebvre & Zingg, 1989; Fehr et al. 1989).

Northern blot analysis revealed that vasopressin and oxytocin transcripts are subject to tissue- and cell-specific differential polyadenylation processes in the rat (Ivell, Schmale, Krisch et al. 1986; Carrazana, Pasieka & Majzoub, 1988; Robinson, Frim, Schwartz & Majzoub, 1988; Zingg, Lefebvre & Almazan, 1988; Fehr et al. 1989). Vasopressin and oxytocin mRNAs from rat hypothalami contained longer poly(A) tails than those from peripheral organs (adrenal gland, testis and ovary). In the case of the mutant vasopressin precursor-encoding transcripts of Brattleboro rats, the length of the poly(A) tail increased during development, being significantly longer in adult than in early postnatal animals (Fehr et al. 1989).

This hitherto poorly understood differential polyadenylation process might be correlated with stimuli evoking up-regulation in the transcription of the vasopressin and/or oxytocin genes. This is supported by recent findings that, in rats, osmotic stress stimulates transcription of the vasopressin gene, yielding concomitantly vasopressin-encoding transcripts with longer poly(A) tails (Carrazana et al. 1988; Zingg et al. 1988). Also, the longer poly(A) tails of the mutant vasopressin mRNA in adult compared with that in infant Brattleboro rats may be related to the more pronounced osmotic stress occurring in the mature animals (Fehr et al. 1989).
On the other hand, triggering of oxytocin gene expression at the onset of the luteolytic cycle in cows does not effect the length of the poly(A) tail (Ivell & Richter, 1984), suggesting that the latter phenomenon per se is not necessarily coupled to increased transcription activities but rather to a tissue-specific process.

Differential polyadenylation processes may also occur during ontogeny of the vasopressin and oxytocin genes. The pig was chosen for a test system since its gestation period is extended (115 days), allowing the collection of sufficient material from different stages of prenatal development. In order to follow up the expression of the two genes at the RNA level, the cDNAs encoding the precursors of porcine lysine vasopressin (Rehbein, Hillers, Mohr et al. 1986) and oxytocin (present study) have been cloned and sequenced. Specific probes have been constructed and used for Northern blot analysis of oxytocin- and vasopressin-encoding mRNAs isolated from microdissected hypothalami from different developmental stages of pigs. The data reveal an increase in the extent of adenylation at the 3' end of both mRNAs throughout development, reaching a maximum in the adult animals.

MATERIALS AND METHODS

Animals

All investigations were performed with German Landrace pigs kept under standard conditions at the Institut für Tierzucht und Tierverhalten at Mariensee, F.R.G. Specimens of both sexes were examined as a pool at different developmental stages: FD 36, n=9; FD 49, n=10; FD 109, n=4; and postnatal day 5, n=2. The adult pigs were all females (n=9).

RNA isolation

Hypothalami were either frozen and stored in liquid nitrogen immediately after the animals were killed or, alternatively, frozen on solid CO₂ and stored at −40 °C, before isolation of the SON and PVN. The latter procedure had the advantage of enriching for magnocellular neurone-derived RNA, although this enrichment was less significant for early prenatal stages, as these neurones had to be excised together with a greater amount of surrounding tissue in comparison with later stages. Excision was achieved by cutting frozen hypothalami transversely in a cryostat until the regions of interest appeared near the surface of the tissue block. The SON/PVN and some of the surrounding tissue, including some of the accessory magnocellular neurones, but not the nucleus suprachiasmaticus, were then excised superficially with a cold needle. The anatomical location of these nuclei was determined by comparison with the Stereotactic Atlas of the Mini Pig's Brain (F. Ellendorff, Mariensee, unpublished).

RNA was extracted from frozen tissue with guanidinium-isothiocyanate (Chirgwin, Przybyla, MacDonald & Rutter, 1979) and purified by CsCl centrifugation and oligo(dT) chromatography.

cDNA cloning and sequence analysis

Total RNA from SON of adult pigs was converted into double-stranded cDNA (Gubler & Hoffman, 1983), inserted by GC base pairing into the PsI site of the pUC 9 vector and transformed into Escherichia coli strain CMK 603. Oxytocin clones were identified by sequential screening, first with a porcine lysine vasopressin cDNA probe (Rehbein et al. 1986) expected to cross-hybridize with oxytocin clones, followed by a probe comprising only the last 220 bp of the 3' end of the vasopressin cDNA which should hybridize specifically with vasopressin clones but not cross-react with oxytocin clones. Colonies were selected that reacted positively with probe 1, but not with probe 2. Screening 2500 colonies yielded 30 oxytocin-specific clones. The longest insert was sequenced (Rehbein et al. 1986) and compared with the previously obtained sequence for lysine vasopressin-encoding cDNA (Fig. 1).

Preparation of the labelled cDNA probes

To construct a vasopressin-specific cDNA probe (vasopressin-3'), a fragment comprising the last 220 bp of the vasopressin cDNA (Fig. 1) plus 16 GC bp plus 8 bp derived from the vector was excised with Nael/HindIII from the original cDNA clone and inserted into pUC 9 previously digested with SmaI and HindIII restriction endonucleases (Boehringer Mannheim GmbH, Mannheim, F.R.G.). An oxytocin-specific cDNA probe (oxytocin-3') was constructed in an analogous fashion from the last 186 bp of the oxytocin cDNA (Fig. 1). The clone Bl 95, specific for the immunoglobulin heavy chain-binding protein (BiP) (Haas & Meo, 1988), was a gift from Dr I. Haas (Institut für Genetik, Universität zu Köln, Cologne, F.R.G.). The respective cDNA inserts were excised from the cloning sites by appropriate enzymes, gel purified and labelled with [α-³²P]dCTP (Amersham Buchler GmbH & Co. KG, Braunschweig, F.R.G.) to approximately 2 x 10⁹ c.p.m./μg (Feinberg & Vogelstein, 1983). No cross-hybridization was observed between the oxytocin-3' and vasopressin-3' cDNA fragments.

Journal of Molecular Endocrinology (1990) 4, 151–158
RNase-protection mapping

$^{32}$P-Labelled vasopressin and oxytocin cRNAs were synthesized in vitro using SP6 polymerase (Promega Corporation, Madison, WI, U.S.A.) (Melton, Krieg, Rebagliati et al. 1984) after subcloning the corresponding cDNA insert into pGem vectors. The template for vasopressin cRNA synthesis was EcoRI linearized pGem 3 vector DNA carrying the complete vasopressin cDNA (Fig. 1). The cRNA derived from the complete oxytocin cDNA sequence (Fig. 1) proved to be less suitable for RNase mapping. The protected fragment appeared in multiple bands, probably due to RNase-resistant secondary structures. The HindIII linearized pGem 4 vector carrying a fragment of the oxytocin cDNA comprising the first 173 bp at the 5' end (Fig. 1) was therefore used for cRNA synthesis. Varying amounts of total RNA were hybridized overnight at 45 °C to 1 ng cRNA labelled to approximately $1\times10^6$ c.p.m./µg (Zinn, DiMaio & Mamiatis, 1983). Following digestion with RNase A (Boehringer Mannheim GmbH; 80 µg/ml for vasopressin and 40 µg/ml for oxytocin) and RNase T1 (Gibco/BRL GmbH, Berlin F.R.G.; 2 µg/ml) for 60 min at 34 °C (vasopressin) and 30 °C (oxytocin), the protected cRNA fragments were separated by electrophoresis on 5% (vasopressin) or 6% (oxytocin) polyacrylamide gels containing 8 M urea. The sizes of the protected fragments were determined after autoradiography by comparison with co-electrophoresed radioactive DNA size markers.

Northern blot analysis

Total RNAs (10 µg) were glyoxalated and separated on a 1-5% (w/v) horizontal agarose gel (McMaster & Carmichael, 1977). Subsequently, the RNA was transferred by capillary blotting onto a Gene Screen Plus membrane (Du Pont de Nemours, Deutschland) GmbH, Bad Homburg, F.R.G.) according to the instructions of the manufacturer, and hybridized in 50% (v/v) formamide at 42 °C under standard conditions (Ivell & Richter, 1984) with the addition of 1 ng probe/ml. Blots were finally washed at 65 °C in 0-2 × SSC (1 × SSC is 0-15 M sodium chloride/0-015 M tri-sodium citrate)/0-1% (w/v) sodium formate at 50 °C.
dodecyl sulphate (SDS) (oxytocin-3' and vasopres¬
sin-3' probes), or at 50 °C in 2×SSC/0-1% SDS
(BiP probe) for 30 min before autoradiography.

If the blots were successively hybridized with two
different probes, the first probe was removed before
the second hybridization by boiling the membranes
for 20 min in 0-1×SSC/1% SDS. Complete re¬
moval of probe was checked by autoradiography.

Poly(A) tails were removed from the mRNA
molecules by incubating 10μg total RNA with

![Figure 2](https://example.com/image2.png)

**Figure 2.** Northern blot analysis of the porcine hypothalamic oxytocin (OT) and lysine vasopressin (VP) precursor mRNAs at different developmental stages. (a) Total RNA from adult porcine hypothalamus was hybridized successively to the VP-specific cDNA fragment and to the OT-specific cDNA fragment. (b and c) Total RNA from different stages (b) before and (c) after removal of poly(A) tails with RNase H was hybridized successively to the OT-specific probe (OT-3') and to the VP-specific probe (VP-3'). Lane 1, total RNA from adult porcine brain cortex; lane 2, total RNA from fetal day (FD) 49 derived from supraoptic and paraventricular hypothalamic nuclei (SON/PVN); lane 3, total RNA from FD 109 SON/PVN tissue diluted fivefold with RNA from brain cortex; lane 4, total hypothalamic RNA from 5-day-old piglets; lane 5, total hypothalamic RNA from adult sows; lane 6, total RNA from FD 109 SON/PVN tissue. As the bands of VP- and OT-encoding mRNAs from FD 109 (lane 6) appeared overexposed compared with those from other stages, total RNA from FD 109 was diluted fivefold (lane 3). The amount of RNA applied to lanes 2–6 corresponds to roughly 1/2, 1/50, 1/100, 1/100 and 1/10 of the given hypothalamus from the respective developmental stage. The co-electrophoresed glyoxalated RNA standards are indicated (b). The indicated size of the deadenylated OT and VP mRNAs (c) was determined by means of the same standards.

*Journal of Molecular Endocrinology (1990) 4, 151–158*
0.2\,\mu g\,\text{oligo (dT)}\,(\text{Pharmacia, Uppsala, Sweden})\,\text{and}\,1\,\text{unit\,RNase\,H}\,(\text{Gibco/BRL\,GmbH})\,\text{as\,described\,previously\,(Ivell\,&\,Richter, 1984)}.

**RESULTS**

The sequence of the cDNA and the predicted prepro-oxytocin precursor presented in Fig. 1 shows considerable homology with the previously published sequences for bovine, rat and human oxytocin precursors (Rehbein et al. 1986). As in calf, rat and man, the degree of homology between the porcine oxytocin and lysine vasopressin precursor mRNAs is lowest in the 3' region. The vasopressin- and oxytocin-specific fragments were therefore constructed from the 3' region of the two cDNAs and probed with porcine total hypothalamic RNA. Northern blot analysis of this RNA fraction isolated from adult animals gave rise to bands of 980 bases for vasopressin- and 900 bases for oxytocin-encoding mRNA (Fig. 2a).

When vasopressin- and oxytocin-encoding transcripts from different developmental stages (FD 36, 49 and 109, postnatal day 5 and adult pig) were analysed, the earliest detectable signals were found at FD 49 (Fig. 2b, lane 2). Even when using the more sensitive RNase-protection protocol (Fig. 3) no positive signals were observed at FD 36. These results are complemented by in-situ hybridization studies of the porcine hypothalamus in which vasopressin mRNA was first detected at FD 40, while neurons showing positive hybridization signals for oxytocin mRNA first appeared at FD 42 (Milewski, Rehbein, Fehr et al. 1988).

Closer inspection of the Northern blots obtained for the vasopressin and oxytocin mRNAs from various developmental stages revealed a rather heterogeneous distribution in size for the two mRNA species, ranging from 840 bases for vasopressin mRNA and 710 bases for oxytocin mRNA at FD 49 to 980 bases (vasopressin) and 900 bases (oxytocin) found in adult animals (Fig. 2b and Table 1). Both hormone-encoding transcripts from FD 49 appear to consist of two populations of different size, with mean lengths of 710 and 780 bases for oxytocin-encoding mRNAs.
and 840 and 890 bases for vasopressin transcripts (Table 1). The differently sized mRNAs at this early stage of neuronal development may well be due to slight differences in the degree of maturation of individual fetuses. An artifact produced by RNA degradation can be excluded since the BiP-encoding mRNA control appeared undegraded in the same preparations (Fig. 4).

Oxytocin and vasopressin mRNAs from the later developmental stages showed increases in length, culminating with the adult stage at which transcripts were extended by some 190 bases (oxytocin) and 140 bases (vasopressin) in comparison with those observed at FD 49. The transcripts from FD 109 and postnatal day 5 were of intermediate length and no significant difference was observed between those two stages.

To establish whether the observed developmental length polymorphisms of the oxytocin- and vasopressin-encoding transcripts were due to differential polyadenylation, total RNA from all stages was digested with RNase H in the presence of oligo(dT). Indeed, after removal of the poly(A) tract, oxytocin and vasopressin mRNAs revealed the same size of 560 and 610 bases respectively, at all stages examined (Fig. 2c). The identical size of the RNase-protected fragments (Fig. 3) further confirms that no sequence differences exist at the different stages examined.

The developmentally related lengthening of poly(A) tails appears to be specific for the oxytocin- and vasopressin-encoding mRNAs. Northern blot analysis of BiP-encoding transcripts revealed no difference in the lengths of mRNAs at all stages examined (Fig. 4).

Comparison of the increase in adenylation of oxytocin- and vasopressin-encoding mRNAs throughout development reveals a more pronounced effect for oxytocin transcripts compared with transcripts for vasopressin (Table 1). At the adult stage the oxytocin mRNA had a poly(A) tail of about 340 residues as opposed to about 370 adenylate residues for the vasopressin mRNA. At FD 49, however, the oxytocin-transcripts carried only 150 or 220 adenylate residues, while the vasopressin transcripts had poly(A) tails of 230 or 280 bases. One explanation might be the delay in the onset of oxytocin gene expression occurring at FD 42 compared with that of the vasopressin gene at FD 40. In the rat, too, in-situ hybridization studies (Fehr et al., 1989) as well as quantitative Northern blot analysis (Almazan et al., 1989) of oxytocin and vasopressin transcripts point to a delayed expression of the oxytocin gene during ontogeny.

**DISCUSSION**

Recently, length polymorphisms of oxytocin and vasopressin mRNAs, as a consequence of differential polyadenylation, have been reported. Oxytocin and

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Approximate length of mRNA (nucleotides)</th>
<th>Approximate length of poly(A) tail (nucleotides)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal day 49</td>
<td>840 and 890</td>
<td>230 and 280</td>
</tr>
<tr>
<td>Fetal day 109</td>
<td>940</td>
<td>230</td>
</tr>
<tr>
<td>Postnatal day 5</td>
<td>920</td>
<td>310</td>
</tr>
<tr>
<td>Adult</td>
<td>890</td>
<td>370</td>
</tr>
</tbody>
</table>

The lengths of the mRNAs were determined by comparison with the co-electrophoresed glyoxalated RNA standards indicated in Fig. 2b. The lengths of the poly(A) tails were calculated from the difference between the lengths of the adenylated and deadenylated mRNAs (Fig. 2b and c).
vasopressin mRNAs from extra-hypothalamic tissues, such as the bovine corpus luteum (Ivell & Richter, 1984) and rat adrenal gland and testes (Ivell et al. 1986), have shorter poly(A) tails than the corresponding hypothalamic transcripts. In rats, the poly(A) tails of hypothalamic vasopressin mRNA were found to be about 250 residues long under normal physiological conditions and to reach about 400 residues in dehydrated animals, when vasopressin synthesis is up-regulated (Carrazana et al. 1988). It has been proposed that the lengthening of the poly(A) tract might provide an additional level for the stimulation of vasopressin gene expression by enhancement of mRNA stability or translational efficiency (Carrazana et al. 1988; Zingg et al. 1988). The mRNA encoding the mutated hypothalamic vasopressin precursor of the Brattleboro rat carrying a single base deletion has a longer poly(A) tail than its wild-type counterpart (Ivell et al. 1986). It has also been shown that the mutant gene transcript is under developmental control, with longer poly(A) tails being present in adults than in early postnatal animals (Fehr et al. 1989). Circadian variations in the length of the vasopressin mRNA have been observed in the parvocellular neurones of the suprachiasmatic nucleus (Robinson et al. 1988). Here, the levels of vasopressin gene transcripts fluctuate in a 24-h cycle, being highest during the daytime, which is reflected in hormone concentrations oscillating in cerebrospinal fluid (Uhl & Reppert, 1986). On Northern blots, two distinctly sized vasopressin mRNA species with poly(A) tails of 240 and 30 residues were observed during the dark phase, while only the long mRNA was found in the light phase (Robinson et al. 1988). Although some data suggest that increased transcription rates and poly(A) tail lengthening are closely coupled processes, there are also experiments where this appears not to be the case. For instance, depletion of serotonin during osmotic stress prevents the increase of vasopressin mRNA levels but not the lengthening of its poly(A) tail (Carter & Murphy 1989).

The increase in poly(A) tail lengths of vasopressin or oxytocin mRNAs in the hypothalamus of the pig could reflect the up-regulation of the respective gene during development. Functional maturation of the oxytocinergic system takes place in the last third of gestation (Sander-Richter, Schams & Ellendorff, 1988). From FD 80 onward, secretion of oxytocin can be triggered by administration of prostaglandin (Sander-Richter et al. 1988). The increase in average vasopressin and oxytocin poly(A) tail length in the developing porcine brain reported here may well have parallels with the functional maturation of the oxytocinergic and vasopressinergic neurones.

While it is not clear by what mechanisms the poly(A) tail length of the respective mRNAs is determined, it has been shown that the activities of nuclear and cytosolic poly(A)-metabolizing enzymes are regulated by phosphorylation (Tsiapalis, 1987). Thus multiple signals from the extracellular environment of the developing neurones, such as cell–cell contact, synaptic input and hormones, might influence the adenylation state and thus the putative translational efficiency of the mRNAs.

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