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Parathyroid hormone-related protein in tissues of the emerging frog (*Rana temporaria*): immunohistochemistry and in situ hybridisation

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ABSTRACT

Using antiserum to human parathyroid hormone-related protein (1–16) [PTHrP(1–16)] we have examined tissues of the common frog (*Rana temporaria*) for the presence of immunoreactive PTHrP (irPTHrP) at the stage of emergence from water to land. irPTHrP was detected in dorsal and ventral stratum granulosum of the skin, in the developing ovary, striated muscle and the choroid plexus epithelium of the brain as well as in the olfactory gland epithelium and olfactory lobe neurons of the brain. In the pituitary and hypothalamus irPTHrP protein could be demonstrated in the median eminence, infundibular stem and principally in the neural lobe and pars distalis of the pituitary with weak reaction in the pars intermedia. In situ hybridisation of the same tissues with an oligonucleotide probe to chicken PTHrP 55–65 clearly showed the presence of mRNA for PTHrP-like molecule in all the tissues containing irPTHrP. There was a major inconsistency in the pituitary in that the highest level of gene expression, assessed by in situ hybridisation, was found in the pars intermedia with only very low expression in the pars distalis and neural lobe and undetectable levels in the infundibular stem and median eminence. These observations suggest that tissues of the frog synthesise a PTHrP-like molecule but that in the pituitary the pars intermedia cells may export the protein to cells in other regions of the pituitary and hypothalamus.

Key words: PTHrP; gene expression; amphibia; development.

INTRODUCTION

Amphibians were the first vertebrates to adapt to a terrestrial environment. By a process of metamorphosis the aquatic larval stages assume a tetrapod form and terrestrial physiology to allow survival in a dry environment and without the support of relatively dense water. Many physiological changes are needed to allow successful life on land, including handling of salt and water balance as well as changes in calcium metabolism for the development of bone as an active tissue responding to mechanical stresses in the absence of the supporting medium of water. An evolutionary development in the amphibians concerned with calcium metabolism is the appearance of the parathyroid gland as a distinct organ, and its secretion, parathyroid hormone (PTH), plays a central role in calcium metabolism by stimulating calcium resorption from bone and inhibiting phosphate excretion via the kidney (Hurwitz, 1989). Of the amphibians, the urodeles are considered to be more primitive because they still lead a mainly aquatic existence whilst the anurans live on dry land as adults, many needing to return to water for egg laying and larval stages of development. In some newts (Urodela) the parathyroid gland develops only in the adult stage (P. K. T. Pang, unpublished observations, 1974) and its secretions elevate serum calcium, partly by actions on the calcium deposits of the endolymphatic sacs, which adjust serum pH as well as calcium. The amphibians are therefore a particularly interesting group in which to study factors which may be concerned in the control of calcium metabolism.

We recently reported the presence of immunoreactive parathyroid hormone-related protein (irPTHrP) in plasma and pituitary gland of a marine

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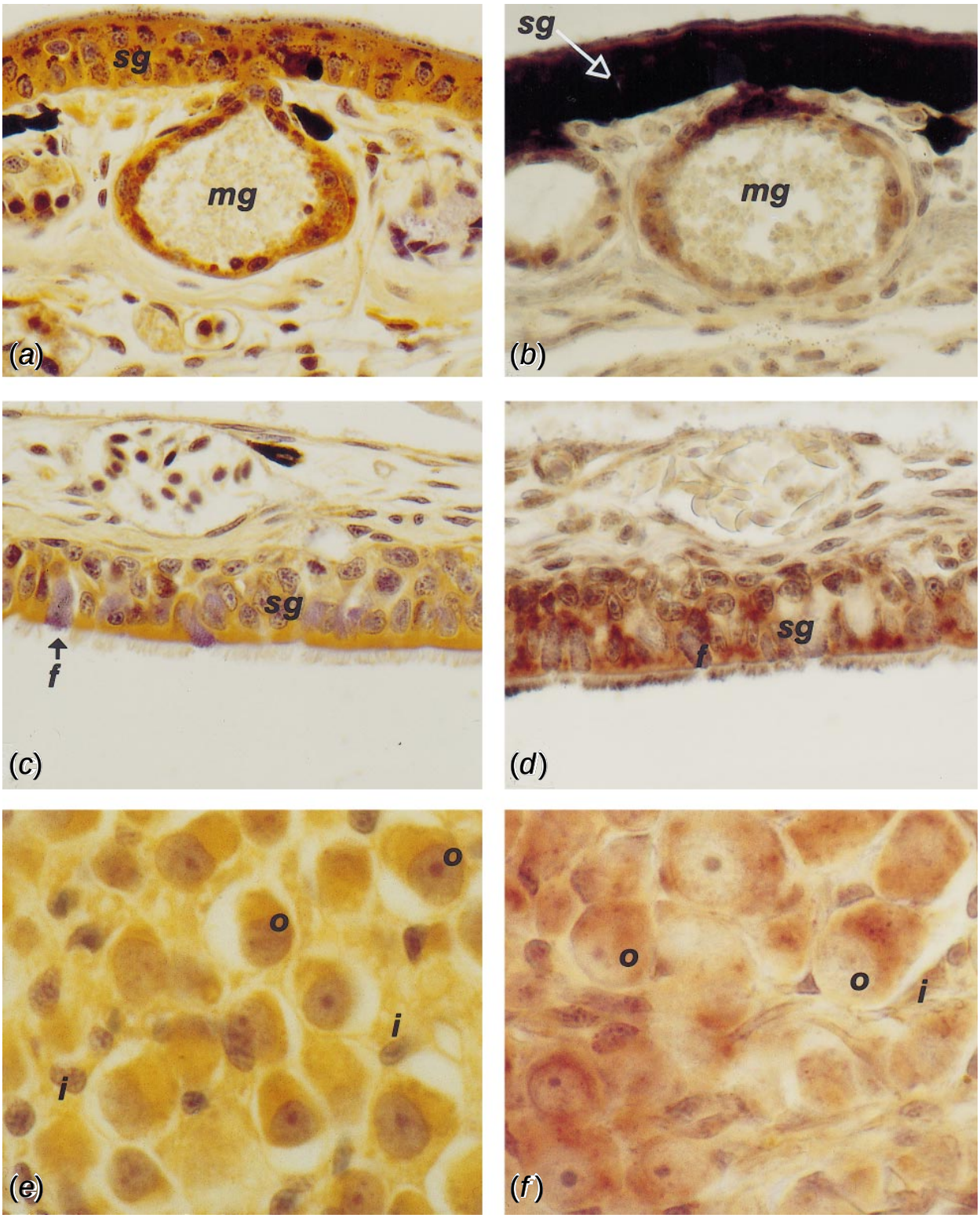

Fig. 1. (*a*) Dorsal skin of the emerging frog immunostained with antibody to human PTHrP(1–16). Intense reaction occurred in the cytoplasm of cells of the stratum granulosum (*sg*) and the epithelial cells of the mucus secreting gland (*mg*), derived from the stratum granulosum. × 200. (*b*) In situ hybridisation of emerging frog dorsal skin with an oligonucleotide probe JD1 to chicken PTHrP. There is hybridisation in the stratum granulosum (*sg*) and mucus gland (*mg*) epithelium, but hybridisation is stronger in the *sg* cells. × 200. (*c*) Ventral skin of the emerging frog showing immunostaining with antibody to human PTHrP(1–16) in the stratum granulosum cells (*sg*), including the ciliated brush border cells, but no staining in the flask cells (*f*). × 200. (*d*) In situ hybridisation of the ventral skin similar to that in (*c*) with the oligonucleotide probe (JD1) to chicken PTHrP, showing gene expression only in the *sg* cells. Both protein and message are less abundant in the ventral skin compared with the dorsal (cf *a* and *b*). × 200. (*e*) Ovary of the emerging frog in which the cytoplasm of both ova (*o*) and interstitial cells (*i*) contain PTHrP immunoreactive with antibody to human PTHrP(1–16). × 250. (*f*) Detection of PTHrP mRNA in the ova (*o*) and interstitial cells (*i*) of the emerging frog ovary with the oligonucleotide probe JD1. × 250.

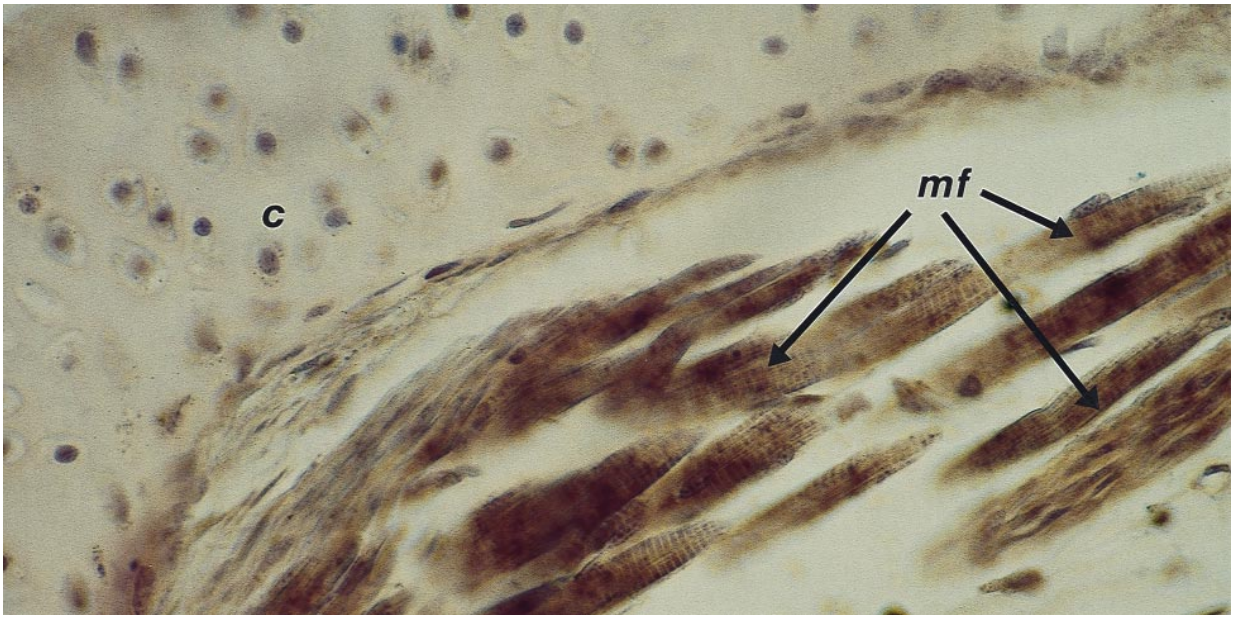

Fig. 2. In situ hybridisation of the emerging frog tissue with the oligonucleotide probe JD1 to chicken PTHrP showing hybridisation to striated muscle (*mf*) but not to adjacent chondrocytes (*c*). ×330.

teleost *Sparus aurata* (Danks et al. 1993) and an elasmobranch fish (Ingleton et al. 1995). In mammals PTHrP mobilises calcium from bone and reduces calcium excretion via the kidney to elevate plasma calcium concentrations (Martin & Suva, 1988). It is produced in mammals in a range of normal tissues, adult (Thiede & Rodan, 1988; Yasuda et al. 1989; Asa et al. 1990; Paspaliaris et al. 1992) and fetal (Moniz et al. 1990; Moseley et al. 1991) and malignant tumours of squamous cell origin as well as breast, kidney and lung cancers (Danks et al. 1989; Campos et al. 1991). Normally in human plasma PTHrP concentrations are low (~2.0 pM/l or less); however, in the sea bream we found that the plasma irPTHrP levels were high and the pituitary gland, during in vitro culture, released significant amounts of PTHrP to produce high medium concentrations (~2.0 nM/l), suggesting that the pituitary may be a major source of plasma irPTHrP in vivo.

It thus seemed particularly pertinent to determine the distribution of PTHrP in an amphibian species since amphibians occupy an important phylogenetic position and show physiological changes associated with adoption of a terrestrial habit. Using immunohistochemistry and in situ hybridisation we have examined tissues of the frog, *Rana temporaria*, for the presence of irPTHrP and PTHrP mRNA in immature frogs at the stage at which they emerge from water to begin terrestrial life (stage XXV, Taylor & Kollros, 1946).

MATERIALS AND METHODS

For this study emerging frogs were collected from private ponds (courtesy Professors I. W. Henderson and R. G. G. Russell). The specimens for examination were collected on the day they emerged from the water, stage XXV (Taylor & Kollros, 1946). Frogs were immersed in MS222 (3-aminobenzoic acid ethyl ester, Sigma) and the anterior region, from the snout to the pectoral girdle, then fixed in sublimated-Bouin Hollande (Kraicer et al. 1967), with the cranial cavity injected with fixative. The fixed tissue was processed through graded alcohols before clearing in xylene and embedding in paraffin wax. Parasagittal sections were cut from the midline at 5 μm and mounted on poly-l-lysine coated slides.

Immunohistochemistry

Immunohistochemistry was performed for the PTHrP antigen (Danks et al. 1989, 1993) with a rabbit polyclonal antibody directed against the first 16 amino acids of human PTHrP (R1133). The peroxidase-antiperoxidase (PAP) method of Sternberger (1974) used primary antibody dilutions of 1:50 and 1:100 with swine antirabbit antiserum (Dako) as the secondary antibody and rabbit PAP complex (Dako). The chromogen utilised was 3,3′-diaminobenzidine (Sigma) and the counterstain was Harris' haematoxylin. This antibody does not cross-react with

parathyroid hormone in mammalian systems and has been used in immunohistochemical studies of teleost tissues as well as those of humans; positive and negative method and antibody specificity controls were included as previously described (Danks et al. 1989, 1993). These included alternating deletion of the antibody layers; the primary antiserum, the secondary antibody and the PAP complex. Also the substitution of nonimmune rabbit serum for the primary antiserum and the overnight preabsorption of the diluted antiserum with hPTHrP(1–34) and hPTH(1–34).

Oligonucleotide probes for in situ hybridisation

A 33-mer sequence oligonucleotide probe taken from the cDNA of chicken PTHrP-5′(GCC CTC ATC TCC ACT GCC AAA ACG GAC AGG GTA)3′-(JD1) was synthesised on an Applied Biosystems Automated DNA Synthesiser Model 392. This sequence is complementary to the region of chicken PTHrP mRNA coding for the amino acid sequence, PTHrP 55–65 (Schermer et al. 1991).

Labelling

All solutions used for probe preparation, prehybridisation and hybridisation were treated with 0.02% DEPC (diethylpyrocarbonate; Sigma). The probes were labelled at the 3′ end using terminal deoxynucleotidyl transferase. The reaction mixture consisted of tailing buffer (GIBCO-BRL: 100 mM potassium cacodylate, pH 7.2, 2 mM $CoCl_2$, 0.2 mM DTT) with 40 units of terminal transferase (GIBCO-BRL), 1 mg PTHrP probe and 25 nmoles of digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim, Germany). The volume was made up to 20 ml with water. The reaction mixture was incubated at 37 °C for 2 to 18 h and stopped with the addition of 2 ml of 200 mM Na_2EDTA. The tailed oligonucleotide was precipitated with 2.5 ml of 4 M LiCl and 75 ml of prechilled 100% ethanol. After mixing well the mixture was chilled at −70 °C for at least 30 min, then centrifuged at 12000 *g* for 10 min and the pellet washed with 50 ml of cold 70% (v/v) ethanol. After removing the ethanol the pellet was dissolved in 100 µl of sterile, redistilled water and stored in aliquots at −20 °C.

In situ hybridisation

The high stringency in situ hybridisation technique was based on that of Danks et al. (1995). Briefly, sections were dewaxed in 2 changes of xylene, followed by 2 washes in 100% ethanol and 2 washes in 90% ethanol. Mercury in the fixative was removed by immersing the slides in 1% iodine in 70% ethanol for 1 min and a subsequent wash in 5% sodium thiosulphate for 1 min. The sections were washed in water for 5 min then 2 × 5 min washes in phosphate buffered saline (PBS). This was followed by a 15 min incubation of the slides in 0.3% Triton X-100 (Sigma) before 2 more 5 min rinses in PBS. They were then postfixed in 4% paraformaldehyde (BDH, Poole, UK) for 5 min and washed twice for 5 min in PBS. Sections were placed in prehybridisation buffer (25% deionised formamide [BDH] in 3 × SSC) for 1 h at room temperature. Hybridisation buffer (20 µl) containing 10 ng of PTHrP labelled probe was applied to the appropriate section. The hybridisation buffer consisted of 3 × SSC, 25% deionised formamide, 0.1% polyvinylpyrrolidone (Sigma), 0.1% Ficoll (Sigma), 1% bovine serum albumin (Sigma), 250 mg/ml salmon sperm DNA (Sigma), 250 mg/ml yeast tRNA (Boehringer Mannheim). The sections were covered with glass coverslips and hybridisation was carried out in a humid chamber for 2 to 18 h at room temperature. Coverslips were removed in 4 × SSC and sections washed for 15 min each in 2 changes of 3 × SSC. They were then washed for 5 min in a modified Tris buffered saline (TBS) consisting of 0.1 M Tris (BDH), 0.15 M sodium chloride (BDH), pH 7.5.

Antibody enhancement

After the wash in TBS the sections were flooded with sheep antidigoxigenin antiserum (Boehringer Mannheim) diluted 1:250 in TBS with 5% normal rat serum. They were incubated in a humid chamber for 1 h at room temperature, after which they were washed 3 times for 5 min in TBS. Alkaline phosphatase-linked donkey antisheep immunoglobulins (Boehringer Mannheim) were applied to the sections at a dilution of 1:500 in TBS with 5% normal rat serum, for 1 h at room temperature. The sections were washed 3 times for 5 min in TBS followed by a single 5 min wash in 0.1 M Tris, 0.1 M sodium chloride, 0.05 M magnesium chloride, pH 9.5. The reaction was visualised using 4-nitroblue tetrazolium chloride (Boehringer Mannheim) and 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer Mannheim) as substrates with 10 mM levamisole (Sigma) to block endogenous alkaline phosphatase activity in the tissues. The sections were counterstained with Mayer's haematoxylin, and mounted with Aquamount (Gurr, Poole, UK).

Controls

Several different control protocols were included to ensure the specificity of the results. (1) Sections were processed as described above but in the absence of labelled probe. (2) Sections were subjected to RNase A (Boehringer Mannheim) treatment (100 μg/ml–200 μg/ml in 2 × SSC for 90 min at 37 °C) before hybridisation. (3) An initial hybridisation was carried with unlabelled probe in a 3-fold excess followed by incubation with labelled probe. (4) Sections were hybridised with a poly d(T) (20-mer) probe (Pharmacia) to assess the total RNA in the sections. (5) Sections were hybridised with a poly d(A) (20-mer) probe (Pharmacia) to act as a sense control for the poly d(T) probe. (6) Sections were hybridised with a 'nonsense' probe. This is a reverse antisense probe (33-mer) to the constant region of the kappa immunoglobulin light chain (a gift from Dr Janice Royds, Department of Pathology, University of Sheffield).

RESULTS

Immunoreactive PTHrP and its precursor mRNA were localised in a number of tissues examined in the anterior region of frogs at the stage of emergence from the aquatic milieu; several of these reactions are illustrated in the following figures. Immunoreactive PTH could not be detected with a rabbit polyclonal antiserum to human PTH(1–34) (Biogenex) and preabsorption of the PTHrP antiserum with hPTH(1–34) did not diminish staining. Figure 1 shows the presence of both protein and mRNA in dorsal and ventral skin and in the ovary. Stratum granulosa cells of the dorsal skin epidermis (Fig. 1*a*, *b*) appeared to contain more immunoreactive PTHrP and stronger hybridisation signal for PTHrP mRNA than in the ventral skin (Fig. 1*c*, *d*), judging by the intensity of staining in the different reactions. Amongst the epithelial cells of the ventral skin, those with microvilli forming a brush border contained immunoreactive PTHrP and PTHrP mRNA, whilst the intercalated flask cells (f) did not react with PTHrP antibody or hybridise with the oligonucleotide probe. In the dorsal skin the epithelial cells forming the mucus-secreting glands, derived from the epidermis, were also positive for irPTHrP and PTHrP mRNA, with the strongest reactions in the neck cells.

In the developing ovary, staining for PTHrP antigen was apparent in the cytoplasm of the ova with a weaker reaction also in interstitial cells (Fig. 1*e*). All these cells expressed PTHrP mRNA, but the ova appeared to have the strongest hybridisation (Fig. 1*f*).

There was a strong hybridisation signal in several other tissues which also reacted with antibody to PTHrP; only the results of in situ hybridisation in these tissues are shown. In Figure 2 striated muscle shows strong hybridisation which appears to be enhanced in the sarcomeres whilst chondrocytes in the adjacent cartilage showed no reaction; a similar pattern was seen with PTHrP immunohistochemistry. In the snout region of the head (Fig. 3*a*) the epithelium of the olfactory organ, composed of columnar cells, contained both PTHrP antigen and mRNA. These cells appeared to be polarised with the nucleus at the pole of the cell adjacent to the basement membrane. PTHrP antigen was evenly distributed throughout the cytoplasm of the epithelial cells but the hybridisation signal was strongest at the apical pole. The olfactory lobe of the brain, seen in the same figure, was made up of numerous small neurons with a large nuclear/cytoplasmic ratio. The cytoplasm of these cells contained PTHrP antigen, with stronger staining in the apices abutting the lumen of the lobe; however, hybridisation to PTHrP mRNA occurred throughout the cytoplasm of these cells. The adjacent cartilage did not react with PTHrP antibody nor with the oligonucleotide probe. All the cells of the choroid plexus of the midbrain contained immunoreactive PTHrP and showed strong hybridisation with the PTHrP probe (Fig. 3*b*).

In cells of the tissues described so far there was a close similarity between expression of PTHrP mRNA and localisation of the PTHrP antigen; however, in the pituitary gland the pattern was different. In Figure 4*a* clear staining with antiserum to PTHrP can be seen in cells of the pituitary pars distalis, the neural lobe, infundibular stem and median eminence, as well as weak staining in cells of the pars intermedia. In contrast to this distribution, hybridisation with the PTHrP probe (Fig. 4*b*) occurred almost exclusively in cells of the pars intermedia where expression was abundant; there was no apparent reaction in other cells of the pituitary or hypothalamus in which the antigen was detected.

No expression of PTHrP mRNA or antigen was seen in the negative control slides.

DISCUSSION

This is the first demonstration of a PTHrP-like peptide in amphibian tissues. The staining with antibody to human PTHrP and in situ hybridisation with a probe

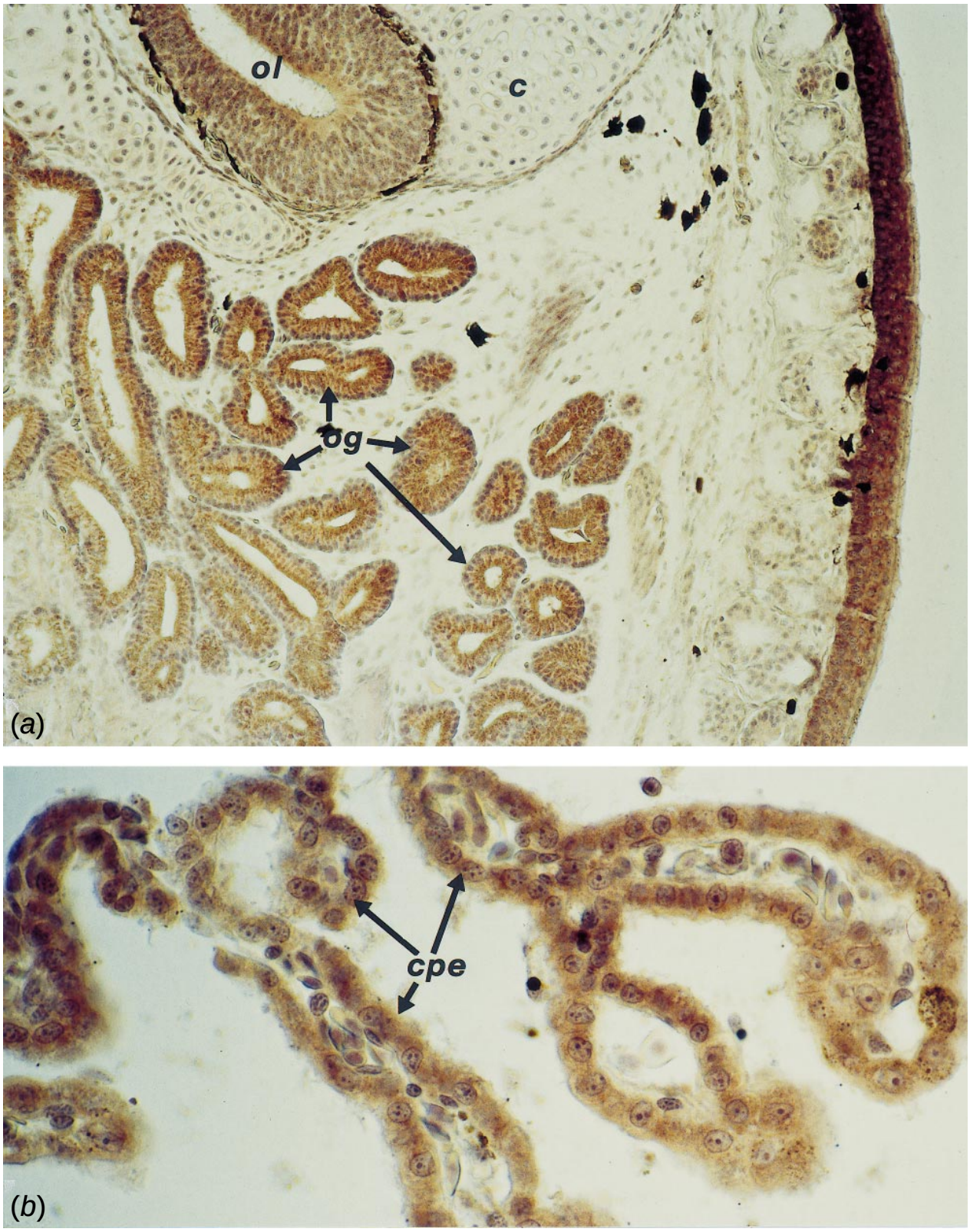

Fig. 3. (*a*) In situ hybridisation of the snout region of the head of emerging frog using an oligonucleotide probe JD1. Hybridisation is apparent in the olfactory gland (*og*) epithelial cells and in the olfactory lobe (*ol*) of the brain as well as in the stratum granulosum of the epidermis. No hybridisation occurred in the cranial cartilage chondrocytes (*c*). ×80. (*b*) Cells of the choroid plexus (*cpe*) of the brain of the emerging frog showing hybridisation with the oligonucleotide probe JD1. ×250.

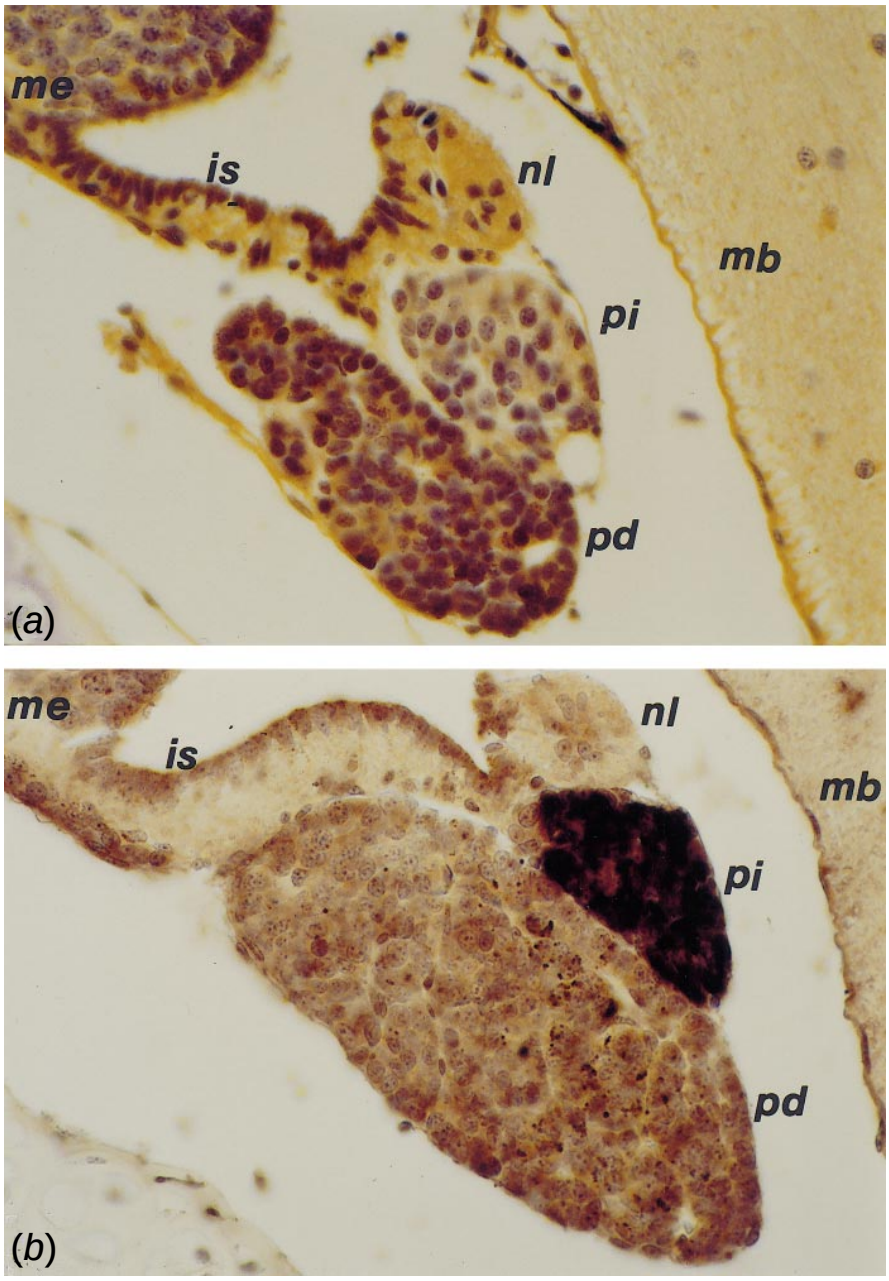

Fig. 4. (*a*) Pituitary of the emerging frog showing immunostaining with antibody to human PTHrP(1–16). Strongest staining occurred in cells of the neural lobe (*nl*), pituitary pars distalis (*pd*), infundibular stalk (*is*) and median eminence (*me*), with weak reaction in cells of the pars intermedia (*pi*). *mb*, midbrain. ×150. (*b*) In situ hybridisation of the pituitary with an oligonucleotide probe to JD1. Although there is a low level of hybridisation in the pars distalis (*pd*) the strongest hybridisation is in the pars intermedia (*pi*) where there appeared to be little immunodetectable protein. (cf *a*). ×150.

selected from the cDNA of chicken PTHrP indicate conservation both of the amino acid sequence and the nucleotide sequence of PTHrP amongst these different vertebrates. These results are consistent with our previous observations that antisera to human PTHrP react with fish tissues and plasma (Danks et al. 1993; Ingleton et al.1995; Devlin et al. 1996). Together these results indicate conservation of PTHrP protein and mRNA, suggesting that it has important biological functions in all these species.

The distribution of the PTHrP antigen in skin, kidney and muscle is similar to that described in rat, human and sheep (Kramer et al. 1991), but in the frog there was differential expression between the dorsal and ventral epidermis. There appeared to be similar layers of cells forming the stratum granulosum in both the ventral and dorsal epidermis of the stage XXV frog, but the dorsal cells showed more intense staining for PTHrP antigen and the mRNA appeared to be extremely abundant in these cells. The difference

between the epithelia may be due to potential dehydration from the upper surface. The outer layers both of dorsal and ventral epithelia do become keritanised, forming a thin stratum corneum (Rosenburg & Warburg, 1978), again suggesting a similarity with human skin in which PTHrP is located in the keratinocytes (Danks et al. 1989, 1995). Epithelial cells of the dorsal skin give rise to the mucus-secreting glands in the frog and the cells of these also stained for PTHrP antigen. The neck cells usually stained more intensely, similar to the epidermal cells; neck cells of hair follicles of fetal rats also show stronger hybridisation than deeper cells (Senior et al. 1991). However, mRNA hybridisation was apparently less abundant in the ventral epithelium indicating that the balance between translation and transcription in the 2 epithelia may be different. In the ventral skin the flask cells, which develop during the later stages of metamorphosis (Rosenberg & Warburg, 1978) do not appear to contain either PTHrP antigen or mRNA, suggesting that it is not produced by and may have no function in these cells. The detection of irPTHrP in these epidermal cells is similar to the distribution in human skin in which the keratinocytes, as opposed to the basal cells, contain PTHrP (Danks et al. 1989). Farquhar & Palade (1964) described cells of the stratum granulosum of frog as showing evidence of secretory activity with well developed rough endoplasmic reticulum and secretory granules; these they presumed to contain mucus but it is possible that they are PTHrP stores.

The kidney tubule cells are sites of PTHrP production in mammalian (Kramer et al. 1991) and elasmobranch species studied (Ingleton et al. 1995) and its presence in these cells as well as in the choroid plexus and epidermis of the emerging frog suggest that it may have a function in hydromineral transport. The choroid plexus maintains hydromineral balance between the blood and the CSF (Cserr, 1971). In mammalian kidney PTHrP interacts with the receptor common both to PTH and PTHrP to cause excretion of phosphate (Orloff et al. 1994) which contributes to raising plasma calcium levels. Whether it has such a function in amphibians remains to be investigated.

The presence of PTHrP antigen and mRNA in the olfactory gland and olfactory lobe of the brain is interesting because it has been shown that smooth endoplasmic reticulum (SER) of olfactory axons terminating in the olfactory lobe of the brain of adult bullfrogs (*Rana catesbiana*) may be responsible for sequestering calcium (Hartter et al. 1987); co-localisation of Ca^{2+}-Mg^{2+}-adenosine triphosphatase (ATPase) also suggests active intraneural regulation of Ca^{2+} levels. There may be an analogy between the olfactory gland and neural lobe location of PTHrP with that in the coronet cells of the saccus vasculosus of fish (Devlin et al. 1996; Ingleton & Danks, 1996) since these cells also have abundant smooth endoplasmic reticulum and appear to contain calcium and Ca^{2+}-ATPase; moreover they are also derived from neural ectoderm.

PTHrP in the ovary of *Rana* suggests it may have functions related to maturation of the ova and possibly in early development of the embryo. If PTHrP is a neurotransmitter as seems possible from its detection in neurons of the olfactory lobe of the frog, in neurons of the dogfish brain (Ingleton et al. 1995) and neuroendocrine tumours (Deftos et al. 1989) then it may also act as a 'prenervous' neurotransmitter (Buznikov, 1984). Such 'prenervous' neurotransmitters, acetylcholine, catecholamines and serotonin, are found in oocytes and pregastrulation embryos of most groups of the metazoa, frequently with several types of neurotransmitter in the same cell (reviewed by Buznikov, 1984). The widespread distribution of PTHrP in mammalian embryonic tissues (Campos et al. 1991; Moseley et al. 1991) and in particular in those such as the liver parenchymal cells from which PTHrP is absent in the adult (Yasuda et al. 1989), suggests that it may have a role in tissue differentiation.

The most intriguing observation of the PTHrP-like protein and its mRNA was in the pituitary in which there was a clear differentiation in abundance between the protein and mRNA. Cells in the pars distalis (PD) stained positively for PTHrP antigen but mRNA expression was very low, whilst the pars intermedia (PI) contained abundant mRNA and very little PTHrP antigen. Moreover the infundibular stem, carrying axons from the brain to the pituitary and the median eminence, reacted with PTHrP antibody but did not hybridise with the PTHrP oligonucleotide probe. These observations suggest that the PI is the principal site of translation of PTHrP mRNA in *Rana* pituitaries but the protein is rapidly transferred to the PD and possibly also, by retrograde transport, to the median eminence and infundibular stem. The presence of mRNA for TGF β in a tissue with the protein for which it encodes detected in adjacent tissues has been noted in developing mouse skin (Lehnert & Akhurst, 1988) and in mouse embryo cardiogenesis (Akhurst et al. 1990) in which TGF β mRNA is proposed to play an important role in heart valve and septa morphogenesis. PTHrP in the frog PI and PD may be similarly organised and possibly be involved in tissue differentiation. The frog PI is richly innervated

directly from the hypothalamus and has vascular supplies only to the periphery and the neurointermedia border (Holmes & Ball, 1997). It is therefore possible that the high levels of PTHrP in the frog PI may partially originate from neurons in the brain since mRNA coding for oxytocin has been found in axons of the hypothalamo-neurohypophysial tract in rats (Jirikowski et al. 1990), but in the frogs we could not detect PTHrP mRNA by in situ hybridisation in the infundibular stem carrying axons from the brain. The frog brain is a potential source of a PTHrP-like protein and its mRNA because staining was intense in the olfactory lobe neurons and choroid plexus. It is interesting, too, that whilst PTHrP antigen was found most abundantly in cells of the PI of the sea bream (Danks et al. 1993), the expression of PTHrP mRNA, detected by in situ hybridisation using the same chicken PTHrP probes, showed only a moderate level of hybridisation (Danks et al. unpublished observations). The principal endocrine cells in the frog PI secrete melanotrophin (MSH) but there are also stellate cells, apparently nonsecretory, ramifying between the secretory cells and connected to a network of stellate cells lying between the endothelium of the superficial capillaries and the PI secretory cells. Further detailed studies of the distribution of PTHrP in the brain and hypothalamus of *Rana* from the neurula stage are needed to determine the possible role of PTHrP as an embryonic inducing factor. Microscopic and ultrastructural immunocytochemistry and in situ hybridisation of PTHrP in the PI and PD in the adult are needed to provide valuable information on the nerves and cells involved in the production of irPTHrP as well as potential interactions between the brain and pituitary.

These observations of PTHrP antigen and PTHrP mRNA in tissues of an amphibian demonstrate the evolutionary persistence of this protein suggesting that it has important functions in several aspects of amphibian biology including tissue development and differentiation, hydromineral balance and neurotransmission.

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