Expression of parathyroid hormone-related protein mRNA in the rat before birth: demonstration by hybridization histochemistry

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ABSTRACT

The ontogeny of parathyroid hormone (PTH) and PTH-related protein (PTHrP) gene expression was studied by hybridization histochemistry in the rat at various stages between implantation and full term. PTHrP mRNA was demonstrable in the early post-implantation trophoblastic giant cells but disappeared from this site before 13·5 days. Localized gene expression, detectable by the in-situ technique, began between 12·5 and 15·5 days in embryonic tissues. The distribution of gene expression suggests that PTHrP may be concerned with the process of implantation. Its widespread, yet clearly localized, distribution in embryonic and fetal tissues is consistent with a paracrine or autocrine function which may relate to the transforming growth factor-β family of growth factors. PTH expression occurred solely in the parathyroid and was detectable in the fetal parathyroid at 13·5 days of gestation.
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INTRODUCTION

It is generally accepted that the hypercalcaemia associated with malignant conditions is explicable, at least to some extent, by the fact that the tumour cells produce and secrete a protein which has an action similar to that of parathyroid hormone (PTH) (Heath, Senior, Varley & Beck, 1990). An 18 kDa PTH-related protein (PTHrP) was purified initially from a lung cancer cell line (Moseley, Kubota, Diefenbach-Jagger *et al.* 1987). Its amino acid sequence was partially determined and subsequently confirmed by cDNA cloning (Suva, Winslow, Wettenhall *et al.* 1987; Mangin, Webb, Dryer *et al.* 1988; Thiede, Strewler, Nissenson *et al.* 1988). The greater part of the structure of the 13 kb human gene is now characterized (Mangin, Ikeda, Dreyer & Broadus, 1989; Suva, Mather, Gillespie *et al.* 1989; Yasuda, Banville, Hendy & Goltzman, 1989; Mangin, Ikeda, Dreyer & Brodus, 1990). Multiple mRNA species, resulting from alternative splicing with variable 5′- and 3′ -untranslated regions, arise from a single gene consisting of seven exons driven by multiple promoters (Mangin *et al.* 1989; Suva *et al.* 1989) and associated with putative regulatory sequences responsive to 1,25-dihydroxyvitamin D_3, glucocorticoids and cyclic AMP (cAMP) (Ikeda, Lu, Weir *et al.* 1989). Proteins 139, 141 and 173 amino acids in length are produced. The somewhat simpler 12 kb rat gene contains four exons and produces a 1·4 kb mRNA from a single promoter, resulting in the generation of a 141 amino acid peptide only (Karaplis, Yasuda, Hendy *et al.* 1990).

In certain situations, both in man (Hammonds, McKay, Winslow *et al.* 1989) and the rat (Zajac, Callaghan, Eldridge *et al.* 1989), post-translational processing can occur. Consequently, PTHrP reaching the circulation could be much shorter.

Although PTH and PTHrP genes seem to have arisen by duplication and thus belong to the same gene family, they show very limited sequence homology which is confined entirely to the N-terminus where eight out of the first 13 amino acids are shared. In this connection, many of the principal biological activities of both proteins reside in their first 34 residues. This applies not only to calcium retention and increased cAMP and phosphate excretion by the kidney (Horiuchi, Caulfield, Fisher *et al.*

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1987; Kemp, Moseley, Rodda *et al.* 1987) but also to their action as smooth-muscle relaxants (Mok, Nickols, Thompson & Cooper, 1989) and (in the case of PTHrP) to its transforming growth factor (TGF)-like properties (Insogna, Stewart, Morris *et al.* 1989).

While increased concentrations of PTHrP can be demonstrated in the serum of many patients with malignancy-associated hypercalcaemia, there is no evidence that it plays a role in the control of calcium homeostasis in normal situations. Its local production is likely to be associated with autocrine and paracrine actions which have not yet been fully delineated. In all mammals so far investigated, the PTH gene seems to be expressed exclusively in the parathyroid glands, while PTHrP mRNA has been demonstrated in a variety of normal peripheral tissues, including rat lactating breast, cultured human keratinocytes, rat stomach and several endocrine glands (Drucker, Asa, Henderson & Goltzman, 1989).

The present report describes the ontogeny of PTH and PTHrP gene expression in the rat demonstrated by hybridization histochemistry using ^{35}S- and ^{32}P-labelled single-stranded RNA probes. The precise temporal and spatial localization of PTHrP provides clues concerning its possible normal biological actions.

MATERIALS AND METHODS

Probes

A 330 bp cDNA for rat PTHrP was used which coded for amino acid 34 to the end of the coding sequence. Northern blots using a rat probe encompassing the sequence used have been published (Thiede, 1989). The cDNA was subcloned into Bluescript (Stratagene, Cambridge, Cambs, U.K.) and its composition verified by plasmid sequencing. The plasmid was used to generate labelled RNA probes; it was linearized either with HindIII ('antisense' transcripts with T7 polymerase) or EcoRI ('sense' transcripts with T3 polymerase). The linearized plasmid templates were purified by phenol/chloroform extraction and ethanol precipitation. Labelled RNA probes were produced by the following method. Five μl 5× transcription buffer (1× = 40 mM Tris–HCl (pH 7·5), 8 mM $MgCl_2$, 50 mM NaCl and 2 mM spermidine (Sigma Chemical Co., Poole, Dorset, U.K.)), 1·5 μl 100 mM dithiothrietol (DTT), 1 μl each of 10 mM ATP, CTP and GTP, 125 μCi [^{35}S]UTP (1000 Ci/mmol; Amersham International plc, Amersham, Bucks, U.K.) or [^{32}P]UTP (800 Ci/mmol; Amersham International plc) and 1 μg template DNA were mixed and made up to a total volume of 24 μl. The appropriate RNA polymerase (1 μl T3, BRL, Paisley, Strathclyde, U.K., or 1 μl T7, Boehringer Mannheim, Livingston, Lothian, U.K.) was added and the whole incubated at 37 °C for 35 min. After this period, a further 1 μl RNA polymerase was added and the incubation continued for a further 35 min. The template was removed by DNase-1 (Amersham International plc) digestion and the probe purified by phenol/chloroform extraction and ethanol precipitation. Yield of transcript was assessed by trichloroacetic acid precipitation of a sample followed by scintillation counting (Maniatis, Fritsch & Sambrook, 1982), and the quality of transcript was assessed by electrophoresis through 4% polyacrylamide gels. For hybridization histochemistry, the mean transcript size was reduced to approximately 150 bases by alkaline hydrolysis (Cox, DeLeon, Angerer & Angerer, 1984) followed by ethanol precipitation, and the probe was stored at −70 °C in 10 mM DTT until use.

A 30 mer oligonucleotide complementary to amino acids 62–71 of rat prepro-PTH was synthesized (Schmelzer, Gross, Widera & Mayer, 1987). The nucleotide sequence used (ACA AGG ACA TTT TCC TCC TTC TTG GTG GGC) is not complementary to prepro-PTHrP mRNA. It was purified by elution from a 10% polyacrylamide gel and 5′ labelled with ^{32}P for hybridization histochemistry. Oligonucleotide (50 ng) was denatured by incubation in 20 mM Tris-HCl (pH 9·5), 1 mM spermidine and 0·1 mM EDTA at 70 °C for 5 min (10 μl), then labelled by the addition of 50 mM Tris–HCl (pH 9·5), 10 mM $MgCl_2$ 5% (v/v) glycerol, 100 μCi [^{32}P]ATP (3000 Ci/mMol; Amersham International plc) and 1·5 μl polynucleotide kinase (Pharmacia, Milton Keynes, Bucks, U.K.) to a total reaction volume of 30 μl. The mixture was incubated at 37 °C for 1 h and the labelled oligonucleotide purified by Sephadex G-25 (Pharmacia) spun column chromatography followed by ethanol precipitation.

Tissue preparation

Rat conceptuses from 7·5 days of age to term were collected (timed from 00.00 h on the night of mating). Tissue for paraffin sectioning was fixed in 4% (w/v) paraformaldehyde in Dulbecco's phosphate-buffered saline (PBS) for 18–24 h, then stored in 0·5 M sucrose in PBS at 4 °C for 2–5 days before dehydrating and wax embedding. Tissue for frozen sections was placed into moulds, covered in OCT (Tissue Tek; Miles Ltd, Slough, Berks, U.K.) and frozen at the surface of a solid CO_2/hexane bath, and the blocks were stored at −40 °C until required.

Paraffin sections were cut at 5 μm, taken up on treated slides (Rentrop, Knapp, Winter & Schweizer, 1986), dewaxed and rehydrated through a graded series of alcohol. For oligonucleotide hybridization histochemistry, the slides were post-fixed in 4% (v/v) gluteraldehyde in 0·1 M phosphate buffer (pH 7·5)/20% (v/v) ethylene glycol for 1 min at 4 °C. They were then rinsed twice in 4 × SSC (1 × = 150 mM HCl and 15 mM sodium citrate; pH 7·0) and prehybridized in hybridization buffer (see below) for 20 min at 42 °C. Slides for RNA probe hybridization histochemistry were rinsed in protease buffer (50 mM Tris–HCl, pH 7·5, plus 5 mM EDTA, pH 8·0) and digested in 125 μg Pronase E/ml (Sigma) in protease buffer at 37 °C for 10 min, after which they were rinsed in PBS containing 0·1% (w/v) glycine and post-fixed in 4% paraformaldehyde in PBS for 10 min. Slides for both techniques were then dehydrated, dried and used immediately for hybridization. A minimum of two embryos per time-point were used.

Frozen sections were cut at 9 μm, thaw mounted onto treated slides (Rentrop *et al.* 1986) and stored on solid CO_2 for 20 min. They were then fixed in 4% paraformaldehyde in PBS for 20 min, dehydrated through a graded series of alcohol, dried and used immediately for hybridization. A minimum of two embryos per time point were used.

Hybridization histochemistry

For the demonstration of PTHrP mRNA, the RNA probe was resuspended at a concentration of 50–100 ng/ml in 0·3 M NaCl, 10 mM Tris–HCl (pH 7·5), 10 mM Na_2HPO_4 (pH 6·8), 5 mM EDTA (pH 8·0), 1 × Denhardt's solution, 1 mg yeast RNA/ml, 50% deionized formamide and 10% (w/v) dextran sulphate. Immediately before use, the mixture was heated to 80 °C, pipetted onto the section, a coverslip applied and the slides were incubated at 50 °C overnight. Slides from each block were hybridized with either the sense or antisense probe, the sense probe acting as the negative control. The coverslips were washed off in hybridization solution made as above but lacking probe, yeast RNA and dextran sulphate. The slides were washed in three changes of this buffer in a shaking bath at 50 °C for 3 h. The slides were then digested with 150 μg RNase A/ml (Sigma) in 0·5 M NaCl, 10 mM Tris–HCl (pH 7·5) and 1 mM EDTA (pH 8·0) for 1 h at 37 °C and then washed twice in 2 × SSC at 65 °C (20 min each wash).

For PTH oligonucleotide hybridization histochemistry, the probe was resuspended at 10 ng/ml in 0·6 M NaCl, 50 mM phosphate buffer (pH 7·2), 40% formamide and 1 μg herring sperm DNA/ml. The mixture was heated to 80 °C for 30 s before use and 40 μl were pipetted onto each slide and a coverslip was applied. Slides were incubated at 42 °C overnight. The coverslips were washed off in 4 × SSC (prewarmed to 45 °C). The slides were then washed for 2 × 20 min in 2 × SSC and for 2 × 20 min in 0·5 × SSC, all at 45 °C, and dehydrated through a graded series of alcohol; ^{32}P-labelled slides were exposed to X-ray film (X-Omat AR; Eastman Kodak, Liverpool, Merseyside, U.K.) overnight to assess the strength of labelling. The PTH probe hybridized specifically and exclusively to embryonic and adult parathyroid tissue. A formal control was provided by hybridization of embryonic tissue with a 30 mer probe for insulin-like growth factor-II (Beck, Samani, Penschow *et al.* 1987) which reacted with embryonic tissues negative to the PTH probe.

All slides were next dipped in K5 (Ilford, Mobberley, U.K.) liquid emulsion diluted 5:3 (w/v) in 1·7% glycerol in water at 45 °C under the appropriate safelight conditions and allowed to dry upright for 2–3 h before being exposed (with dessicant) at 4 °C for 3–10 days (^{32}P) or 7–14 days (^{35}S). They were then developed by sequential immersion in 160 *g* D19 developer/l (Eastman Kodak) (for 4 min), 1% (v/v) acetic acid (for 1 min) and 20% Hypam fixer (Ilford) (for 4 min), and then washed in tap water, fixed for 10 min in 10% neutral-buffered formol–saline, stained with haematoxylin, dehydrated, cleared and coverslips added. The slides were examined using bright- and dark-field microscopy.

RESULTS

To identify the cells responsible for PTHrP mRNA production, sections of uteri from non-pregnant and pregnant rats on days 7·5, 8·5, 9·5, 10·5, 11·5 and 12·5 of gestation were subjected to hybridization histochemistry with the PTHrP riboprobe, the PTH oligonucleotide probe and the relevant sense controls. In addition, parasaggital and some transverse sections of fetuses and placentae on days 13·5, 15·5, 16·5, 18·5, 19·5 and 20·5 were similarly examined. As a further positive control for PTHrP expression, cryostat and paraffin sections of lactating breast (Thiede & Rodan, 1988) were prepared as above. A positive signal was seen with the antisense probe only and hybridization was absent in the non-lactating breast.

PTHrP was clearly demonstrable in both polar and mural trophoblast cells at 7·5 days of gestation (Fig. 1), while all other embryonic and extra-embryonic tissues as well as the adjacent decidua and other visible maternal components of the uterine

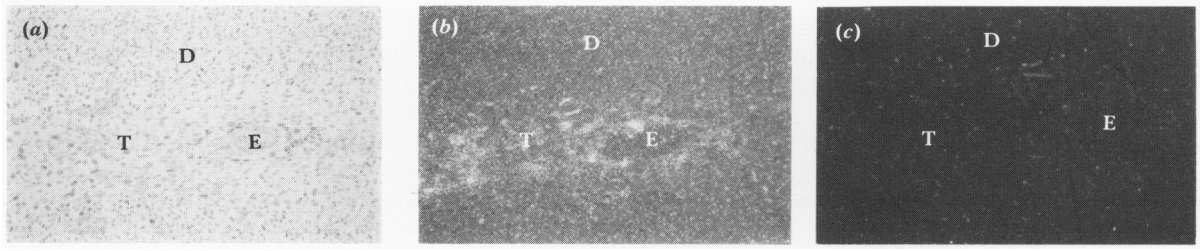


FIGURE 1. Oblique section through rat egg cylinder at 7·5 days of gestation, showing hybridization with parathyroid hormone-related protein probe in trophoblastic giant cells (T). The cells of the embryo (E) and the decidua (D) are negative. (a) Bright field, (b) dark field, (c) dark field 'sense' probe control (× 150).

implantation segment were negative. Hybridization remained strongly localized in the trophoblastic giant cells until 12·5 days of gestation. Not all these cells gave a positive hybridization reaction, although the majority did so, particularly in the early stages of development. From 9·5 days onwards, positive cells appeared more or less deeply placed in the decidual tissue. These were almost certainly invading trophoblastic cells, although the distinction from decidual cells could not be made with certainty (Fig. 2). At 10·5 days, the invading trophoblastic cells were particularly prominent in the decidua capsularis. The presence of PTHrP mRNA in the secondary trophoblastic giant cells contrasted dramatically with its absence in their immediate precursors – the cells of the ectoplacental cone (Fig. 3), as well as from other embryonic and extra-embryonic tissues. At 11·5 and 12·5 days, scattered cells in the mesometrium and extra-decidual uterine stroma gave a positive reaction, although not as markedly as did the trophoblasts. The origin of these cells is not clear and they were not numerous. By 13·5 days, the PTHrP gene ceased to be expressed by trophoblastic giant cells, although these were still prominent placental constituents. Close examination of the extra-embryonic membranes at various stages

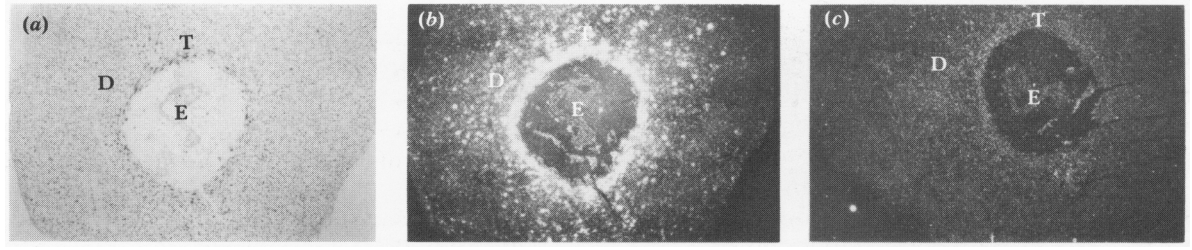


FIGURE 2. Transverse section through rat egg cylinder at 9·5 days of gestation probed with parathyroid hormone-related protein 'antisense' and 'sense' probes. Trophoblastic giant cells (T) give a positive hybridization reaction as do many cells more deeply placed in the decidua (D). The latter are probably invading trophoblastic cells. The cells of the embryo (E) are negative. (a) Bright field, (*b*) dark field, (*c*) dark field sense probe control (× 60).

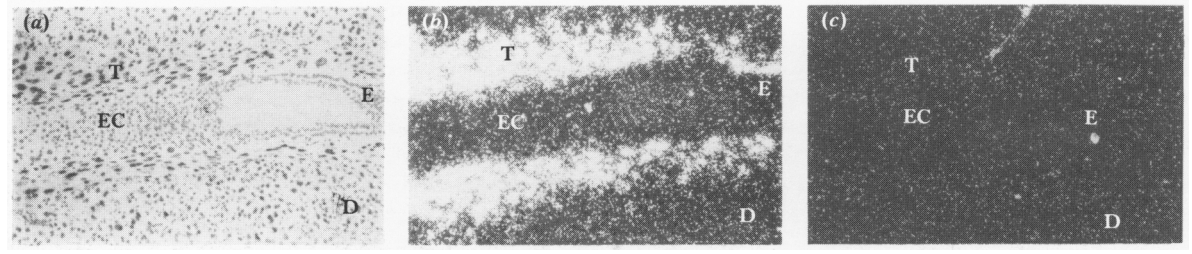


FIGURE 3. Longitudinal section through portion of rat egg cylinder at 8·5 days of gestation hybridized with parathyroid hormone-related protein probe. There is strong hybridization in the trophoblastic cells (T) but none at all in their precursors, the cells of the ectoplacental cone (EC). Decidual (D) and embryonic (E) cells are also negative. (*a*) Bright field, (*b*) dark field, (*c*) dark field sense probe control (× 150).

after 13·5 days revealed no evidence of further detectable production of PTHrP mRNA.

PTH mRNA was not demonstrable in extra-embryonic tissues at any stage of development; the specificity of the PTH probe used was ascertained by its strong hybridization solely to cells of the adult rat parathyroid gland (Fig. 4).

Transverse sections of embryos through the third dorsal pharyngeal pouch (which forms the single pair of parathyroid glands present in the rat) at 13·5 and 15·5 days did not reveal the expression of PTHrP. On the other hand, distinct labelling with the PTH probe was demonstrable in these parathyroid anlagen at both stages. No other embryonic tissue examined gave hybridization with the PTH oligonucleotide probe.

PTHrP was not demonstrable in embryonic tissues examined before 15·5 days. At 15·5 days, the epithelial cells of the dental lamina hybridized strongly, although the surrounding oral epithelium, including the lip furrow band, was negative. The positive reaction persisted strongly throughout gestation and, when the enamel organs were formed, gene expression in both the outer epithelial layer and the ameloblastic layer was clearly seen, but the dental papilla and the cells of the stellate reticulum were not distinguishable from background (Fig. 5). At term, hybridization persisted more strongly in the outer epithelial layer.

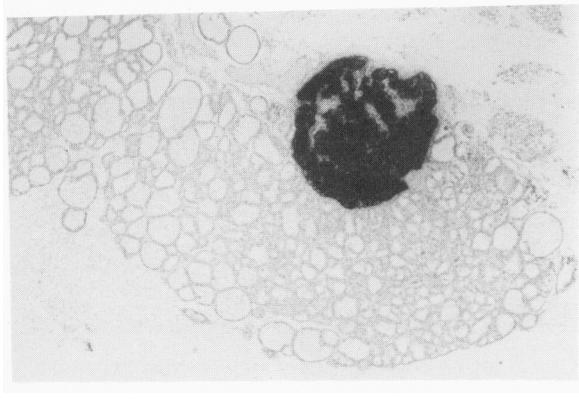


FIGURE 4. Section through adult rat thyroid and parathyroid glands hybridized with parathyroid hormone oligonucleotide. A strong positive reaction over the parathyroid gland is apparent. The thyroid gland is negative (× 100).

Other ectodermal epithelial organs also reacted strongly; hair follicles were positive at 18·5 days, and sections through the vibrissae at 19·5 days showed that the cells involved were those of the outer root sheath (Fig. 6). Often the cells in the neck region of the follicles showed the strongest signal. By 20·5 days, the keratinocytes, particularly in the head region, also gave a positive reaction but this was not as marked as in the hair follicles. The nasal epithelia and regions of the membranous labyrinth (derived from the epithelium of the otic placode) also showed distinct gene expression.

Some cells of endodermal origin also hybridized with the probe. At 15·5 days, when the lungs are at the glandular stage of development, there was widespread hybridization in the epithelial lining of the bronchi, the majority of the cells showing strong gene expression, although occasional negative areas were seen (Fig. 7). During the subsequent canalicular and alveolar stages, positive cells become restricted in their distribution and, in late gestation, relatively isolated patches of positive epithelium could be seen distributed throughout the bronchial tree. Lung mesenchyme remained negative throughout (Fig. 8). In the sections examined, hybridization did not occur in the gut or in its endodermal derivatives (liver and pancreas), neither was there any evidence of mRNA production in the bladder epithelium.

Specific mesodermal derivatives also gave positive results with the PTHrP probe. At 16·5 days, the perichondrium in various parts of the developing skeletal system hybridized strongly (Fig. 9) but expression fell away as development proceeded and only a weak positive reaction was demonstrable at term. In the nose and ear, hybridization occurred in the nasal capsule and bony labyrinth in addition to

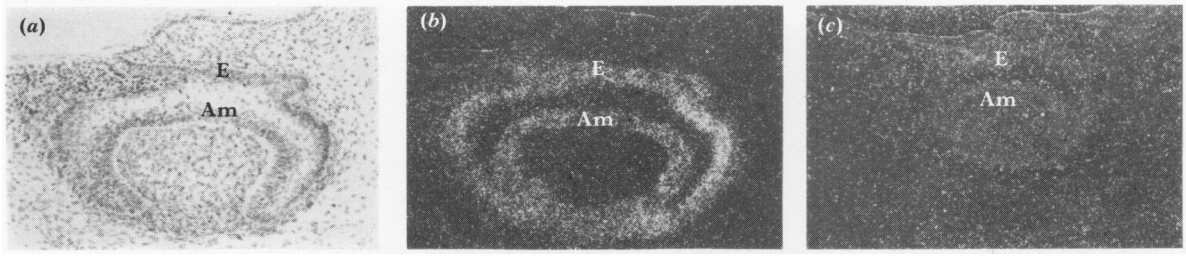


FIGURE 5. Rat tooth bud at 19·5 days of gestation hybridized with parathyroid hormone-related protein probe. Strong gene expression is seen in outer epithelial (E) and ameloblastic layers (Am) of the enamel origin. (*a*) Bright field, (*b*) dark field (× 275), (*c*) dark field 'sense' probe control (× 150).

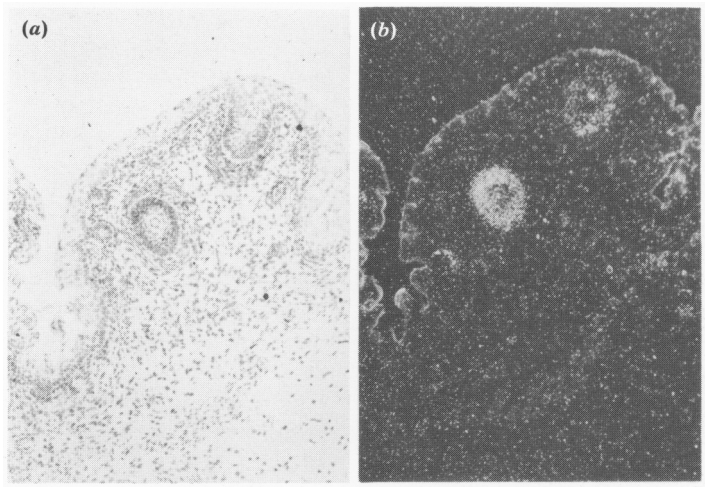
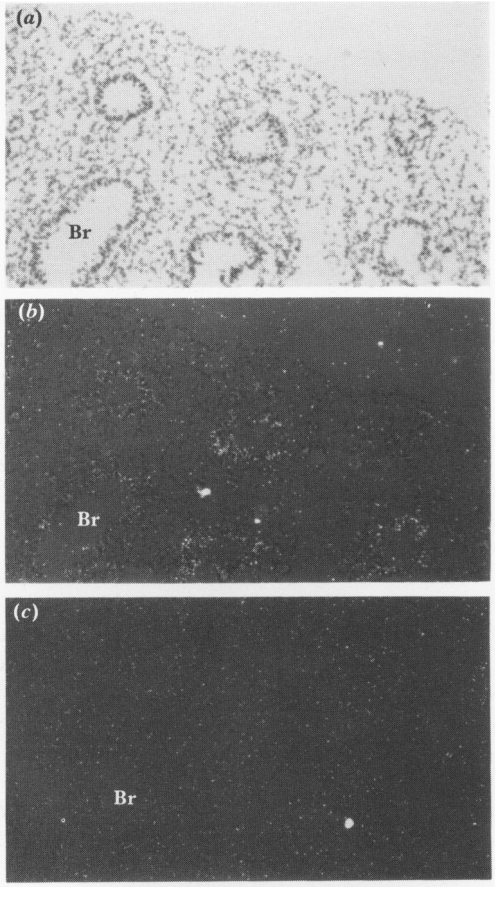


FIGURE 6. Section through rat skin at 19·5 days of gestation hybridized with parathyroid hormone-related protein 'antisense' RNA probe. A positive reaction is seen in the outer root sheath of the vibrisseal hair follicles. At this stage the signal over the skin keratinocytes is at background level only. (*a*) Bright field, (*b*) dark field (× 160).



its previously stated occurrence in the associated ectoderm of the nasal epithelium and membranous labyrinth.

In general, neural tube and neural crest derivatives were not positive; however, a systematic analysis of the central nervous system was not performed, and occasionally a positive region of nervous tissue was apparent (e.g. an area of the medulla oblongata at 18·5 days).

DISCUSSION

We have presented an overview of the expression of the gene for PTHrP during rat ontogenesis. Transcription in embryonic tissues appears to begin after 13·5 days, although examination of a complete set of serial sections would be necessary to exclude with certainty the presence of small areas of earlier gene expression. By 15·5 days, specific mRNA was clearly demonstrable and restricted to well-defined developing organs. Some compact well-circumscribed anlagen may not have been picked up in the sample of sections examined, but our procedure will have

FIGURE 7. Section through rat lung at 15·5 days of gestation hybridized with a parathyroid hormone-related protein 'antisense' probe. The majority of epithelial cells lining the branches (Br) are distinctly positive. (*a*) Bright field, (*b*) dark field, (*c*) dark field sense probe control (× 215).

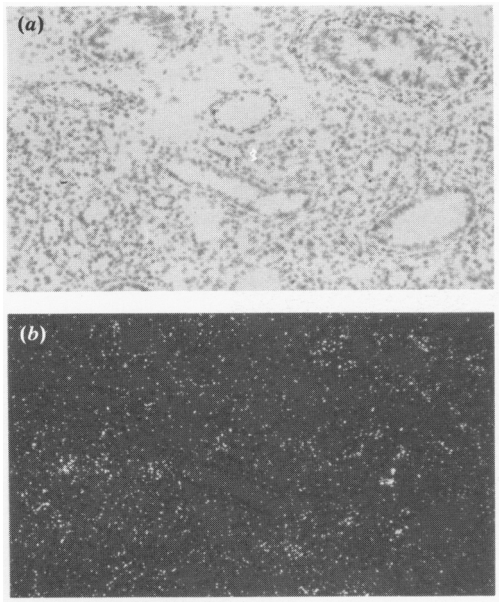


FIGURE 8. Section through rat lung at 18·5 days of gestation hybridized with a parathyroid hormone-related protein 'antisense' probe. Scattered areas of positive epithelium are seen in isolated patches of bronchial epithelium. (*a*) Bright field, (*b*) dark field (×225).

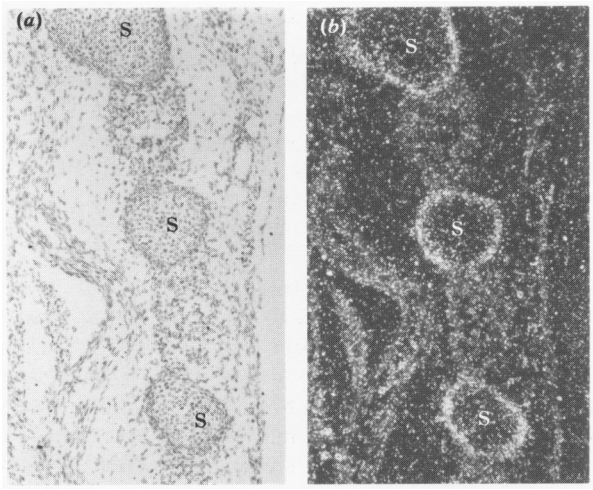


FIGURE 9. Section through sternal region of rat embryo at 16·5 days of gestation hybridized with a parathyroid hormone-related protein 'antisense' probe. Strong hybridization in the perichondrium of the sternum (S) is apparent. (*a*) Bright field, (*b*) dark field (×225).

demonstrated the main sites of activity. The restricted expression of the PTHrP mRNA contrasts with the report of the wide tissue distribution of PTHrP in human fetuses studied by immunocytochemistry (Moniz, Burton, Malik *et al.* 1990).

The presence of PTHrP mRNA in hair follicles and skin keratinocytes correlates with the immunocytochemical demonstration of the protein at these sites (Martin, Allan, Caple *et al.* 1989), although location of the message in the outer root sheath of the follicle rather than in the inner sheath was observed. The other developing organs in which gene expression has been demonstrated have not previously been described as sites of PTHrP production. Its localization in the dental lamina and enamel organ is particularly striking and, taken together with transient expression in perichondrium and periosteum, may be related to local calcium metabolism. Presence of the mRNA in the epithelium of developing bronchi is interesting as squamous cell bronchial carcinoma is particularly associated with hypercalcaemia of malignancy (Heath *et al.* 1990); it seems reasonable to assume that such tumours originate from the scattered positively hybridizing cells present in the bronchial epithelium at birth. In this connection, it would be interesting to determine whether gene expression occurs in the cells of milk lines, because breast carcinoma is similarly associated with hypercalcaemia and there is evidence of PTHrP secretion in lactating mammary tissue (Thiede & Rodan, 1988). Some developmental role in maturation of the central nervous system cannot be ruled out. A hybridization histochemistry study of PTHrP reported by Weir, Brines, Ikeda *et al.* (1990) describes gene expression in the neurones of the cerebral cortex, hippocampus and cerebellar cortex of the adult rat but does not deal with prenatal development.

It has been reported (Insogna *et al.* 1989) that PTHrP possesses TGF-β-like qualities; thus it induces epidermal growth factor-dependent transformation of NRK 49F cells on soft agar and increases the biosynthesis of fibronectin in human dermal fibroblasts. Other workers (Centrella, Canalis, McCarthy *et al.* 1989) claim that PTHrP may regulate osteoblastic cell activity in part by modulating the effects of locally produced TGF-β in bone, and it is interesting that, in the mouse, hybridization histochemistry studies of TGF-β-1 mRNA (Lehnert & Akhurst, 1988) indicate a similar distribution of PTHrP in tooth, hair follicle and perichondrial osteocytes. Furthermore, the presence of murine TGF-β-2 has been documented in perichondrium and the epidermis of mature and late-fetal hair follicles (Pelton, Nomura, Moses & Hogan, 1989).

Other growth factors may also be involved. In particular, most dental and bone morphogenic proteins belong to the TGF-β super family (Wozney, Rosen, Celeste *et al.* 1988), and their presence in precartilaginous mesenchyme (Lyons,

Pelton & Hogan, 1989) as well as possibly in perichondrium and tooth anlagen could be influenced by the presence of PTHrP. The temporally and spatially co-ordinated expression of several members of the TGF-β super family in controlling the development of various mouse organs has been clearly documented (Lyons *et al.* 1989). A careful study of the exact timing of PTHrP expression in this context is, therefore, likely to be rewarding.

The biological significance of PTHrP production by the trophoblast is unclear. From work on sheep (Rodda, Kubota, Heath *et al.* 1988), it has been suggested that PTHrP maintains the positive materno/fetal calcium gradient (Fisher, 1986) required for the growth of the fetal skeleton. PTH does not appear to have this effect; PTHrP(1–34) also is ineffective, whereas PTHrP(1–84) and PTHrP(1–141) are active (Abbas, Pickard, Rodda *et al.* 1989). The molecular basis of this action is not known. In the sheep, as in the rat, PTHrP has been demonstrated in the placenta during early gestation. PTHrP has been detected in the fetal parathyroid of sheep (Loveridge, Caple, Rodda *et al.* 1988; Martin *et al.* 1989; Abbas, Pickard, Illingworth *et al.* 1990) and cattle (Connor, Drees, Thurston *et al.* 1989; Abbas *et al.* 1990) and in the human fetus (Moseley, Hayman, Danks *et al.* 1991). Following in-utero parathyroidectomy in sheep, the materno/fetal calcium gradient is abolished and is not restored by infusion of PTH into the fetal circulation; however, infusion of PTHrP increases fetal calcium (Abbas *et al.* 1989; Martin *et al.* 1989). Although secretion of PTHrP has been demonstrated in a rat parathyroid cell line *in vitro* (Zajac *et al.* 1989), we were unable to demonstrate expression of PTHrP at a detectable level in the anlagen of the parathyroid at 13·5 and 15·5 days of gestation. In contrast, the developing parathyroid is expressing high levels of PTH mRNA during this period. In the rat, we could demonstrate no detectable expression of PTHrP in the trophoblast after 13 days, suggesting that this aspect of fetal metabolism differs between rodents and other mammals.

The localization of PTHrP mRNA described here suggests alternative or additional roles for the hormone. In the immediate post-implantation period, before the fetal stage of development, there is no special need for calcium transport to facilitate skeletal development. Instead, the role of PTHrP as a smooth-muscle relaxant (Mok *et al.* 1989) may be of primary importance. Inhibition of myometrial contraction is an important factor in allowing implantation to take place and preventing the expulsion of the nidus. It is well known that progesterone produces this effect and it has been postulated that, in the human, local progesterone production by the trophoblast supplements its systemic action at the uterine site of implantation (Finn & Porter, 1975). In the rat, the trophoblast secretes progesterone in only the last few days before term (Finn & Porter, 1975) but PTHrP resulting from translation of its mRNA in the trophoblastic giant cells is ideally located, topographically, to act in a paracrine manner and inhibit the myometrium at implantation sites. PTH has been shown to inhibit both the frequency and magnitude of either spontaneous or oxytocin-stimulated uterine contractions (Shaw & Pang. 1985). Mok *et al.* (1989) have shown that PTHrP, like PTH, is an effective relaxant of smooth muscle and that the effect of PTHrP, like PTH, could be inhibited by a molar excess of PTH antagonist. Together with other evidence, this suggests that both hormones interact with the same receptor.

In addition to its effects on smooth muscle, PTHrP increases plasminogen activator activity in osteogenic sarcoma cells (Kemp *et al.* 1987). Furthermore, inhibition of plasminogen activators by antibodies blocks the invasive properties of human trophoblastic cells in an invasion assay devised by Yagel, Parhar, Jeffrey & Lala (1988). It is conceivable, therefore, that PTHrP may also have a role in trophoblast invasion during the period in which the decidua is actively penetrated by the giant cells. Though highly speculative, this suggestion corresponds in timing with gene expression by the trophoblastic giant cells.

In a recent study (Chan, Strewler, King & Nissenson, 1990), both spontaneous and induced differentiation of the parietal endoderm phenotype in various lines of embryonal carcinoma cells were reported as being temporally associated with the expression of adenylate cyclase-coupled receptors which are common to PTH and PTHrP. In addition, low levels of PTHrP mRNA were produced by the cell lines as the first step in the differentiation sequence. Our studies are probably not sensitive enough to detect the low levels of PTHrP mRNA produced by the parietal yolk sac cells; however, they support the suggestion that PTH-responsive adenylate cyclase might constitute a response system by which PTHrP regulates the differentiation and/or function of parietal endoderm. Thus PTHrP secreted in large amounts by adjacent trophoblastic giant cells would act in a paracrine manner to maintain the parietal yolk sac cells in the same way as exogenous PTHrP enhances retinoic acid-induced differentiation of F9 cells to parietal endoderm (Chan *et al.* 1990). It is interesting that the rat parietal yolk sac disappears at day 16 of gestation (Amoroso, 1958; Mosman, 1987), 3 days after the trophoblastic giant cells cease to express PTHrP mRNA.

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REFERENCES

Abbas, S. K., Pickard, D. W., Illingworth, D., Storer, J., Purdie, D. W., Moriz, C., Dixit, M., Caple, I. W., Ebeling, R. R., Rodda, C. P., Martin, T. J. & Care, A. D. (1990). Measurement of parathyroid hormone-related protein in extracts of fetal parathyroid glands and placental membranes. *Journal of Endocrinology* **124**, 319–325.

Abbas, S. K., Pickard, D. W., Rodda, C. P., Heath, J. A., Hammonds, R. G., Wood, W. I., Caple, I. W., Martin, T. J. & Care, A. D. (1989). Stimulation of ovine placental calcium transport by purified natural and recombinant parathyroid hormone-related protein (PTHrP) preparations. *Quarterly Journal of Physiology* **74**, 549–552.

Amoroso, E. C. (1958). Placentation. In *Marshall's Physiology of Reproduction*, vol. 2, pp. 267–269. Ed. A. S. Parkes. London: Longmans.

Beck, F., Samani, N. J., Penschow, J. D., Thorley, B., Tregear, G. W. & Coghlan, J. P. (1987). Histochemical localisation of IGF-I and -II mRNA in the developing rat embryo. *Development* **101**, 175–184.

Centrella, M., Canalis, E., McCarthy, T. L., Stewart, A. F., Orloff, J. J. & Insogna, K. L. (1989). Parathyroid hormone-related protein modulates the effect of transforming growth factor-beta on deoxyribonucleic acid and collagen synthesis in fetal rat bone cells. *Endocrinology* **125**, 199–208.

Chan, S. D. H., Strewler, G. J., King, K. L. & Nissenson, R. A. (1990). Expression of a parathyroid-like protein and its receptor during differentiation of embryonal carcinoma cells. *Molecular Endocrinology* **4**, 638–646.

Connor, C. S., Drees, B. M., Thurston, A., Forte, L., Hermreck, A. S. & Hamilton, J. W. (1989). Bovine parathyroid tissue: a model to compare the biosynthesis and secretion of parathyroid hormone and parathyroid hormone-related peptides. *Surgery* **106**, 1057–1062.

Cox, K. H., DeLeon, D. V., Angerer, L. M. & Angerer, R. C. (1984). Detection of mRNAs in sea urchin embryos by in situ hybridization using asymmetric RNA probes. *Developmental Biology* **101**, 485–502.

Drucker, D. J., Asa, S. L., Henderson, J. & Goltzman, D. (1989). The parathyroid hormone-like peptide gene is expressed in the normal and neoplastic human endocrine pancreas. *Molecular Endocrinology* **3**, 1589–1595.

Finn, C. H. & Porter, D. G. (1975). *The Uterus*. London: Paul Elek.

Fisher, D. A. (1986). The unique endocrine milieu of the fetus. *Journal of Clinical Investigation* **78**, 603–611.

Hammonds, R. G. Jr., McKay, P., Winslow, G. A., Diefenbach-Jagger, H., Grill, V., Glatz, J., Rodda, C. P., Moseley, J. M., Wood, W. I. & Martin, T. J. (1989). Purification and characterization of recombinant human parathyroid hormone-related protein. *Journal of Biological Chemistry* **264**, 14806–14811.

Heath, D. A., Senior, P. V., Varley, J. M. & Beck, F. (1990). Parathyroid-hormone-related protein in tumours associated with hypercalcaemia. *Lancet* **335**, 66–69.

Horiuchi, N., Caulfield, M. P., Fisher, J. E., Goldman, M. E., McKee, R. L., Reagan, J. E., Levy, J. J., Nutt, R. F., Rodan, S. B., Schofield, T. J., Clemens, T. L. & Rosenblatt, M. (1987). Similarity of synthetic peptide from human tumor to parathyroid hormone *in vivo* and *in vitro*. *Science* **238**, 1566–1568.

Ikeda, K., Lu, C., Weir, E. C., Mangin, M. & Broadus, A. E. (1989). Transcriptional regulation of the parathyroid hormone-related peptide gene by glucocorticoids and vitamin D in a human C-cell line. *Journal of Biological Chemistry* **264**, 15743–15746.

Insogna, K. L., Stewart, A. F., Morris, C. A., Hough, L. M., Milstone, L. M. & Centrella, M. (1989). Native and a synthetic analogue of the malignancy-associated parathyroid hormone-like protein have *in vitro* transforming growth factor-like properties. *Journal of Clinical Investigation* **83**, 1057–1060.

Karaplis, A. C., Yasuda, T., Hendy, G. N., Goltzman, D. & Banville, D. (1990). Gene-encoding parathyroid hormone-like peptide: nucleotide sequence of the rat gene and comparison with the human homologue. *Molecular Endocrinology* **4**, 441–446.

Kemp, B. E., Moseley, J. M., Rodda, C. P., Ebeling, P. R., Wettenhall, R. E., Stapleton, D., Diefenbach-Jagger, H., Ure, F., Michelangeli, V. P., Simmons, H. A., Raisz, L. G. & Martin, T. S. (1987). Parathyroid hormone-related protein of malignancy: active synthetic fragments. *Science* **238**, 1568–1570.

Lehnert, S. A. & Akhurst, R. J. (1988). Embryonic expression of TGF beta type-1 RNA suggests both paracrine and autocrine mechanisms of action. *Development* **104**, 263–273.

Loveridge, N., Caple, I. W., Rodda, C., Martin, T. J. & Care, A. D. (1988). Further evidence for a parathyroid related protein in the fetal parathyroid glands of sheep. *Quarterly Journal of Experimental Physiology* **73**, 781–784.

Lyons, K. M., Pelton, R. W. & Hogan, B. L. M. (1989). Pattern of expression of murine Vgr-1 and BMP-20 RNA suggests that transforming growth factor-β-like genes co-ordinately regulate aspects of embryonic development. *Genes and Development* **3**, 1657–1668.

Mangin, M., Ikeda, K., Dreyer, B. E. & Broadus, A. E. (1989). Isolation and characterization of the human parathyroid hormone-like peptide gene. *Proceedings of the National Academy of Sciences of the U.S.A.* **86**, 2408–2412.

Mangin, M., Ikeda, K., Dreyer, B. E. & Broadus, A. E. (1990). Identification of an upstream promotor of the human parathyroid hormone-related peptide gene. *Molecular Endocrinology* **4**, 851–858.

Mangin, M., Webb, A. C., Dryer, B. E., Posillico, J. T., Ikeda, K., Weir, E. C., Stewart, A. F., Bander, N. H., Milstone, L., Barton, D. E., Francke, U. & Broadus, A. E. (1988). Identification of a cDNA encoding a parathyroid hormone-like peptide from a human tumor associated with humoral hypercalcemia of malignancy. *Proceedings of the National Academy of Sciences of the U.S.A.* **85**, 597–601.

Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory.

Martin, T. J., Allan, E. H., Caple, I. W., Care, A. D., Danks, J. A., Diefenbach-Jagger, H., Ebeling, P. R., Gillespie, M. T., Hammonds, G., Heath, J. A., Hudson, P. J., Kemp, B. E., Kubota, M., Kukreja, S. C., Moseley, J. M., Ng, K. W., Raisz, L. G., Rodda, C. P., Simmons, H. A., Suva, L. J., Wettenhall, R. E. H. & Wood, W. I. (1989). Parathyroid hormone-related protein: isolation, molecular cloning and mechanism of secretion. *Recent Progress in Hormone Research* **45**, 467–502.

Mok, L. L., Nickols, G. A., Thompson, J. C. & Cooper, C. W. (1989). Parathyroid hormone as a smooth muscle relaxant. *Endocrine Reviews* **10**, 420–436.

Moniz, C., Burton, P. B. J., Malik, A. N., Dixit, M., Banga, J. P., Nicolaides, K., Quirke, P., Knight, D. E. & McGregor, A. M. (1990). Parathyroid hormone-related peptide in normal human fetal development. *Journal of Molecular Endocrinology* **5**, 259–266.

Moseley, J. M., Hayman, J. A., Danks, J. A., Alcorn, D., Grill, V., Southby, J. & Horton, M. A. (1991). Immunohistochemical detection of parathyroid hormone-related protein in human fetal epithelia. *Journal of Clinical Endocrinology Metabolism*. (In Press.)

Moseley, J. M., Kubota, M., Diefenbach-Jagger, H., Wettenhall, R. E., Kemp, B. E., Suva, L. J., Rodda, C. P., Ebeling, P. R., Hudson, P. J., Zajac, J. D. & Martin, T. J. (1987). Parathyroid hormone-related protein purified from a human lung cancer cell line. *Proceedings of the National Academy of Sciences of the U.S.A.* **84**, 5048–5052.

Mosman, H. W. (1987). *Vertebrate Fetal Membranes*. London: Macmillan.

Pelton, R. W., Nomura, S., Moses, H. L. & Hogan, B. L. M. (1989). Expression of transforming growth factor β2 RNA during murine embryogenesis. *Development* **106**, 759–767.

Rentrop, M., Knapp, B., Winter, H. & Schweizer, J. (1986). Aminoalkylsilane-treated glass slides as support for *in situ* hybridization of keratin cDNAs to frozen tissue sections under varying fixation and pretreatment conditions. *Histochemical Journal* **18**, 271– 276.

Rodda, C. P., Kubota, M., Heath, J. A., Ebeling, P. R., Moseley, J. M., Care, A. D., Caple, I. W. & Martin, T. J. (1988). Evidence for a novel parathyroid hormone-related protein in fetal lamb parathyroid glands and sheep placenta: comparisons with a similar protein implicated in humoral hypercalcaemia of malignancy. *Journal of Endocrinology* **117**, 261–271.

Schmelzer, H. J., Gross, G., Widera, G. & Mayer, H. (1987). Nucleotide sequence of a full-length cDNA clone encoding preproparathyroid hormone from pig and rat. *Nucleic Acids Research* **15**, 6740.

Shaw, R. L. & Pang, P. K. T. (1985). Effects of parathyroid hormone on uterine contraction *in vivo*. In *Current Trends in Comparative Endocrinology* p. 1031. Eds B. Lofts & W. N. Holmes. Hong Kong: Hong Kong University Press.

Suva, L. J., Mather, K. A., Gillespie, M. T., Webb, G. C., Ng, K. W., Winslow, G. A., Wood, W. I., Martin, T. J. & Hudson, P. J. (1989). Structure of the 5′ flanking region of the gene encoding human parathyroid hormone-related protein (PTHrP). *Gene* **77**, 95–105.

Suva, L. J., Winslow, G. A., Wettenhall, R. E., Hammonds, R. G., Moseley, J. M., Diefenbach-Jagger, H., Rodda, C. P., Kemp, B. E., Rodriguez, H., Chen, E. Y., Hudson, P. J., Martin, T. J. & Wood, W. I. (1987). A parathyroid hormone-related protein implicated in malignant hypercalcemia: cloning and expression. *Science* **237**, 893–896.

Thiede, M. A. (1989). The mRNA encoding a parathyroid hormone-like peptide is produced in mammary tissue in response to elevations in serum prolactin. *Molecular Endocrinology* **3**, 1443–1447.

Thiede, M. A. & Rodan, G. A. (1988). Expression of a calcium-mobilizing parathyroid hormone-like peptide in lactating mammary tissue. *Science* **242**, 278–280.

Thiede, M. A., Strewler, G. J., Nissenson, R. A., Rosenblatt, M. & Rodan, G. A. (1988). Human renal carcinoma expresses two messages encoding a parathyroid hormone-like peptide: evidence for the alternative splicing of a single-copy gene. *Proceedings of the National Academy of Sciences of the U.S.A.* **85**, 4605–4609.

Weir, E. C., Brines, M. L., Ikeda, K., Burtis, W. J., Broadus, A. E. & Robbins, R. J. (1990). Parathyroid hormone-related peptide gene is expressed in the mammalian central nervous system. *Proceedings of the National Academy of Sciences of the U.S.A.* **87**, 108–112.

Wozney, J. M., Rosen, V., Celeste, A. J., Mitsock, L. M., Whitters, M. J., Kriz, R. W., Hewick, R. M. & Wang, E. A. (1988). Novel regulators of bone formation: molecular clones and activities. *Science* **242**, 1528–1534.

Yagel, S., Parhar, R. S., Jeffrey, J. J. & Lala, P. K. (1988). Normal non-metastatic human trophoblast cells share *in vitro* invasive properties of malignant cells. *Journal of Cellular Physiology* **136**, 455– 462.

Yasuda, T., Banville, D., Hendy, G. N. & Goltzman, D. (1989). Characterization of the human parathyroid hormone-like peptide gene: Functional and evolutionary aspects. *Journal of Biological Chemistry* **264**, 7720–7725.

Zajac, J. D., Callaghan, J., Eldridge, C., Diefenbach-Jagger, H., Suva, L. J., Hudson, P., Moseley, J. M., Michelangeli, V. P. & Pasquini, G. (1989). Production of a parathyroid hormone-related protein by a rat parathyroid cell line. *Molecular and Cellular Endocrinology* **67**, 107–112.