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Human Parathyroid Hormone as a Secretory Peptide in Milk of Transgenic Mice

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Abstract In a transgenic mouse model we have targeted the expression of recombinant human parathyroid hormone (hPTH) to the mammary gland yielding hPTH as a secretory, soluble peptide in milk. A 2.5 kb upstream regulatory sequence of the murine whey acidic protein (WAP) directed the expression of the hPTH cDNA in a fusion gene construct (WAPPTHSV2) containing the SV40 small t-antigen intron and polyadenylation site in the 3′ end. Established lines of transgenic mice secreted hPTH to milk in concentrations up to 415 ng/ml. Recombinant hPTH recovered from the milk was purified by HPLC and shown to be identical to hPTH standard as analyzed by SDS-PAGE followed by immunoblotting. Expression of the WAPPTHSV2 was limited to the mammary gland as analyzed by polymerase chain reaction (PCR) and Southern blot of reversed transcribed mRNA from different tissues. hPTH is an important bone anabolic hormone and may be a potentially important pharmaceutical for treatment of demineralization disorders such as osteoporosis. We present the transgenic animal as a possible production system for hPTH. © 1995 Wiley-Liss, Inc.

Key words: human parathyroid hormone, transgenic mice, mammary gland, secretory peptide, osteoporosis

Human parathyroid hormone (hPTH) is a peptide hormone produced in the parathyroid gland as a prepro-polypeptide of 115 amino acids. It is processed to the biologically active hPTH of 84 amino acids prior to secretion from the gland into the circulation. hPTH exerts its physiological effects through receptor binding to the target cells in bone and renal tubuli and is the principal regulator of the calcium homeostasis in the body [Potts et al., 1982; Rosenblatt, 1984] in addition to inducing bone formation [Slovik et al., 1986; Reeve et al., 1980; Tam et al., 1982; Ejersted et al., 1993]. This makes hPTH a potentially important pharmaceutical for the treatment of osteoporosis, a pathological condition characterized by a reduction in the amount of bone leading to skeletal fragility and fractures after minimal trauma [Riggs and Melton, 1986].

A number of studies indicate that hPTH has an inherent instability when expressed as a recombinant hormone in bacteria and yeast [Born et al., 1987; Breyel et al., 1984; Rabbani et al., 1988; Høgset et al., 1990a,b; Gabrielsen et al., 1990].

Purification of biologically active heterologous proteins from milk of transgenic animals may represent an efficient way for production of hormones as pharmaceuticals [Janne et al., 1992]. Regulatory sequences from milk proteins genes represent a logical choice for expression of foreign secretory peptides in the mammary gland [Yu et al., 1989; Pittius et al., 1988; Reddy et al., 1991; Gunzburg et al., 1991].

In this study transgenic mice were made by pronuclear microinjections of the gene construct WAPPTHSV2, consisting of the 5′ regulatory sequence of the murine whey acidic protein gene fused to the hPTH cDNA and the SV40

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t-antigen polyadenylation signal. These mice produced and secreted intact hPTH to the milk.

MATERIALS AND METHODS

Materials

Restriction enzymes and other DNA-metabolizing enzymes were obtained from New England Biolabs (Beverly, MA). Chemically synthesized PTH (1–84) (Sigma Chemical Co, St. Louis, MO) and recombinant hPTH from yeast [Gabrielsen et al., 1990] were used as reference preparations. ^{125}I-antirabbit-IgG was from Amersham Corp. (Arlington Heights, IL). Generation and characterization of hPTH antiserum in cock reacting predominantly with the sequence 44–68 in the hormone and anticock-IgG from rabbit have been described earlier [Gautvik et al., 1979].

Construction of WAPPTHSV2 Fusion Gene and Production of Transgenic Mice

The plasmid construction were performed according to standard recombinant DNA methods [Maniatis et al., 1982]. The cloning procedure of cDNA encoding the prepro-polypeptide for hPTH has been described elsewhere [Høgset et al., 1990a,b]. The 2.5 kb upstream regulatory murine WAP sequence was kindly provided by Prof. B. Groner, Ludwigs Institute for Cancer Research, Bern, Switzerland, in the plasmid pPolyIIIWAP. Vector DNA sequencing of the junction between the WAP regulatory sequence and hPTH cDNA, the whole hPTH cDNA and the junction between hPTH cDNA and the SV40 polyadenylation sequence was performed using the Sequenase Kit (United States Biochemical Corporation, Cleveland, OH) according to the supplier's manual. The production of transgenic mice was performed as described [Hogan et al., 1986].

Detection of the hPTH Transgene Using PCR and Southern Blot Analyses

Tail tissue (2–3 mm) from 10-day-old offspring were prepared for PCR and analyzed for the presence of the transgene [Shizhong and Evans, 1990]. PCR was carried out as described [Saldi et al., 1986] using a 25-mer oligonucleotide (*p1*) hybridizing to the WAP/hPTH junction (Fig. 1) with the sequence 5′-TCACTTGCCTGACCAGGTACCCGGG-3′ containing 17 bases upstream of the hPTH cDNA sequence, and a 25-mer oligonucleotide (*p2*) hybridizing to

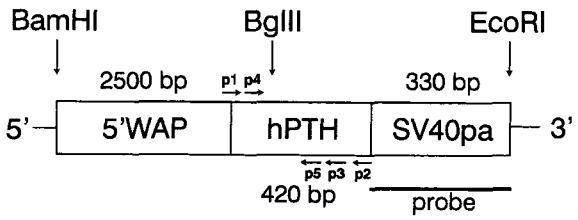

Fig. 1. Schematic presentation of the fusion gene construct WAPPTHSV2 used in production of transgenic mice. 5'WAP: Upstream regulatory sequences of the murine whey acidic protein gene. hPTH: the cDNA encoding hPTH. SV40pa: the SV40 small t-antigen intron and polyadenylation signal. bp: basepairs. The size of the fusion gene components is shown. p1–p5: Location of oligonucleotides used as primers in PCR analysis of DNA and RNA extracted from tissue of transgenic mice. Direction of DNA synthesis is indicated by horizontal arrows. Probe: Location of the ^{32}P-labelled hybridization probe used in Southern blot analysis of DNA extracts from tail tissue for the detection of transgenic mice. Relevant sites for restriction endonucleases are shown. BamHI and EcoRI were used for excising the insert used for the microinjection experiments. BglII was used for digestion of genomic DNA in Southern blot analysis.

the 25 bases in the 3′ end of the hPTH cDNA (Fig. 1) with sequence 5′-CCTCTAGAGCAGAACTCTGACAATA-3′. The PCR product had a theoretical size of 437 bp.

Southern blot analyses [Southern, 1975] of DNA from the same tissue were carried out for confirmation of the PCR results. DNA was prepared from 2 cm finely minced tail tissue after proteinase K digestion by extraction and precipitation in a nucleic acid extractor model 340A (Applied Biosystems, Foster City, CA) according to User Bulletin, Issue No. 21, March 2, 1988. The DNA was digested, electrophorized and blotted to Gene Screen Plus membranes (NEN, Boston, MA) and hybridized with ^{32}P-labelled probe (multi-prime labelling, Amersham Corp.) according to the supplier's instructions.

hPTH mRNA Analysis by PCR and Southern Blot

Total RNA was isolated as described [Chomczynski and Sacchi, 1987], and treated with RQ1 RNase-free DNase (Promega Corp., Madison, WI) for 30 min at 37°C according to supplier's manual prior to the PCR reaction. Reverse transcription cDNA synthesis on total RNA was carried out with an hPTH specific primer (*p3*) complementary to the 3′ coding region (Fig. 1) (5′-CGGGTTCTCTAGACTGGGATTTAGCTTTAGTTA-3′), using 1 μl total RNA (>1 μg/μl), as described [Yang et al., 1989]. After 1:50 dilution of *p3* PCR was carried out on resulted cDNA with primer *p4* (Fig. 1) (5′-CATTGTAT-

GTGAAGATGATACCTGC-3′) and primer *p5* (Fig. 1) (5′-CATTCACATCAGCTTTGTCTGCC-TC-3′). tRNA (2 μg) was added to hPTH mRNA and included as positive control. Expected length of PCR amplified DNAs are 336 bp (Fig. 3). Southern blot analysis of the resulted cDNA was carried out as described above.

Milk Collection

Milk was collected on the fifth and tenth day post partum from anaesthesized animals as described [Simons et al., 1987] using an electrical pumping device of our own design which made possible collection up to 2 ml mouse milk per individual. Milk samples were stored frozen prior to analysis.

Intact hPTH Measurements in Milk by Two-Site Chemiluminometric Immunoassay

Milk samples were diluted 1:50 with distilled water and subjected to a two-site chemiluminometric immunoassay for intact hPTH (1–84) (*Magic Lite* Intact PTH Immunoassay, Ciba Corning Diagnostics Corp., Medfield, MA) employing an N-terminal anti-hPTH (1–34) antibody and a mid-region monoclonal anti-hPTH (44–68) antibody. The assay has a lower detection limit in serum of 1.4 ng/l. Four control samples consisting of milk from normal mice received 0, 1, 10, and 50 ng hPTH/50 ml and the hormone recovery was compared to 10 ng hPTH/50 ml added to distilled water. The hormone recovery of hPTH added to mouse milk was: 91 ± 5.1% (2 SD) (n = 3), and the values obtained from unknown samples were not corrected.

Partly Purification and Measurements of Intact hPTH From Blood

Approximately 1 ml blood was recovered from anaesthezised mice by canylation of their hearts using a heparinized syringe for measurements of intact hPTH. The plasma (420 μl) were adjusted to pH 1 by 1 volume of 2 M HCl, 10% (v/v) formic acid, 2% (v/v) trifluoroacetic acid (TFA), and 2% (w/v) NaCl and centrifuged. hPTH was concentrated and partly purified from the supernatant using Sep-Pak cartridges (Waters Chromotography Division, Millipore, Milford, MA). The cartridges were equilibrated with 10 ml of acetonitrile (ACN) containing 0.1% TFA, followed by 10 ml 0.1% TFA. TFA (0.1%) was added to the supernatant to a total volume 10 ml. This was passed through the cartridge several times in both directions with the help of syringes fixed on both ends of the cartridge. Thereafter, it was washed with 10 ml 0.1% TFA. hPTH was eluted with 40% ACN containing 0.1% TFA. The eluate was freeze-dried and dissolved in 500 ml of sample-buffer (0.05 M phosphate buffered saline, pH 7.4, with 0.1 g/100 ml bovine serum albumin) for two-site chemiluminometric immunoassay for intact hPTH (1-84). The hormone recovery of hPTH added to mouse blood was: 89 ± 5.3% (2 SD) (n = 3), and the values obtained from unknown samples were not corrected. The specificity of the immunoassay towards PTH from other species is not specified by the manufacturer.

Purification of hPTH From Mouse Milk

Two milliliters of milk from transgenic mice received an equal volume of water and were centrifuged and cooled on ice. The fat was removed and 1 volume of a solution containing 2 M HCl, 10% (v/v) formic acid, 2% (w/v) NaCl, and 2% (v/v) trifluoroacetic acid, was added the diluted and defatted milk and centrifuged. The supernatant was adjusted to pH 2 by 10 M NaOH, centrifuged, filtered, and subjected to reverse phase HPLC.

A linear gradient of 35–65% eluant B was run for 35 min using a Nova-Pak C_{18} column (8 × 100 mm) from Waters Instruments and Millipore (Milford, MA). Eluant A, 0.115% trifluoroacetic acid in MilliQ water; eluant B, 0.085% trifluoroacetic acid in 70% aqueous acetonitrile. The flow rate was 1 ml/min. The eluates were monitored by ultraviolet detection at 220 nm and fractions collected for further SDS-PAGE gel electrophoretic analysis. Milk samples from non-transgenic control mice were treated identically.

Polyacrylamide Gel Electrophoresis and Immunoblotting

Polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) was performed as described [Laemmli, 1970]. Samples were solubilized in equal volumes in a buffer containing 0.1 M Tris-HCl, pH 7.5, 17% glycerol, 4% SDS, 0.05% bromphenol blue, and 2% 2-mercaptoethanol and incubated in boiling water bath for 5 min before loading on a 15% gel. Electrophoresis was carried out for 2–3 h at 600 V constant voltage.

Proteins fractionated by SDS-PAGE were transferred electrophoretically to Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore) using the buffers of Towbin et al. [1979] and a "Semi-dry Electroblotter model B" from ANCOS APS, Vig, Denmark. The transfer was complete after 2 h at 0.2 A constant current.

For staining of total proteins in milk the filters were routinely soaked in 0.1% Coomassie-R in 50% methanol for 10 min, followed by destaining in 50% methanol, 10% HAc three times for 5 min, and air drying of the filters.

For antibody probing the stained filters were soaked in methanol for a few seconds and rinsed in water for 5 min, and blocked by incubating the filters in phosphate buffered saline with 5% non-fat dry milk for 1 h at room temperature. Antibody incubations and washes were performed according to Towbin et al. [1979]. Cock anti-hPTH antiserum that reacts with epitopes within amino acid residues 44–68 (C-terminal) [Gautvik et al., 1979] was used (dilution 1:8,000) as the primary antibody and rabbit anticock-IgG (dilution 1:1,000) as the secondary antibody. As tertiary antibody we used ^{125}I-antirabbit-IgG from donkey. Autoradiography was performed overnight at −70°C with Hyperfilm-MP RPN.6 from Amersham Corp. and intensifying screen.

RESULTS

Construction of WAPPTHSV2 Fusion Gene

The hPTH cDNA encoding the entire prepro-hPTH [Høgset et al., 1990a,b] including its start and stop codons was isolated as a 420 bp KpnI/SalI fragment from the plasmid puc19prepro-PTH [Høgset et al., 1990a,b] and inserted in the 3′ end of the WAP regulatory sequence subsequent to KpnI/SalI digestion of the plasmid pPolyIIIWAP containing 2.5 kb murine WAP upstream regulatory sequences and a polylinker. The WAPPTH fusion gene was isolated by SalI/partial XhoI digestion, and by blunt end ligation it was fused in its 3′ end to the SalI/partial ScaI digested fragment from the plasmid pSVCAT containing the SV40 t-antigen 3′ intron with the polyadenylation signal making the plasmid pWAPPTHSV1 of 6.5 kb. By HpaI/partial XbaI digestion 150 bp prokaryotic vector sequences was removed from pWAPPTHSV1 between the SV40 DNA and the hPTH cDNA, and the resulting plasmid of 6.35 kb was designated pWAPPTHSV2. Correct hPTH cDNA sequence, including upstream and downstream junctions, was confirmed by DNA sequencing (not shown). The EcoRI/BamHI 3.25 kb fragment WAPPTHSV2 containing the WAP regulatory sequence-hPTH cDNA-SV40 polyadenylation signal fusion gene was isolated and used for the microinjection experiments (Fig. 1).

Production of Transgenic Mice and Detection of Transgenics by Southern Blot and PCR Analyses

Approximately 400 mouse zygotes were successfully microinjected with WAPPTHSV2 and reimplanted in foster mothers. Of 58 live offsprings six (mice 6, 32, 33, 37, 47, and 54) carried the injected construct genomically integrated as shown by analysis of extracted tail tissue DNA by PCR (Fig. 2A, lanes 2–7) and Southern blot analysis as exemplified by mouse 47 (Fig. 2B, lane 2), respectively. PCR analysis of the transgenic mice resulted in a band comparable in size to the band from pWAPPTHSV2 (Fig. 2A) and compatible with the theoretical

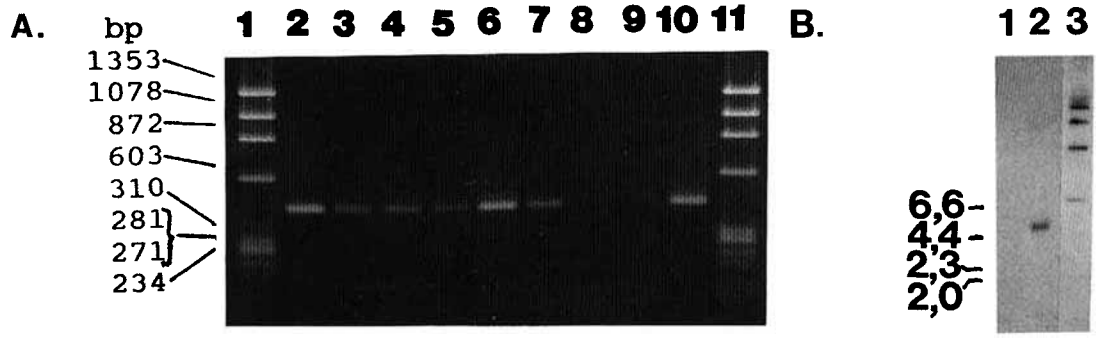

Fig. 2. **A:** PCR analysis of DNA extracted from tail tissue of transgenic mice. *Lanes 1* and *11:* Molecular size marker, HaeIII digested ϕX174. *Lanes 2–8:* Mice 6, 32, 33, 37, 47, 54, and 12. *Lane 9:* Non-injected control mouse. *Lane 10:* pWAPPTHSV2. **B:** Southern blot analysis of DNA extracted from tail tissue of transgenic mice, shown in lane 2 (exemplified by founder mouse no. 47). Lane 1: Non-transgenic control mouse. The DNA from control and transgenic mice were digested by BglII. Lane 3: the expression plasmid pWAPPTHSV2, undigested. Molecular size marker is given in kilobases. Hybridization probe is shown in Figure 1. Exposure time was 17 h.

size of 437 bp using primers *p1* and *p2*, with *p1* covering 17 bases of the 3′ WAP sequence (Fig. 1). Southern blot analysis of BglII digested genomic DNA from tail tissue of transgenic mice resulted in bands of about 6 kb, with a variation in size from 5.4–6.6 kb among the individuals analyzed (as exemplified by mouse 47 in Fig. 2B, lane 2). A weak hybridization signal was seen in the high molecular weight area, probably resulting from incomplete digestion of the genomic DNA (Fig. 2B, lane 2). Uninjected control mice were negative both in PCR (Fig. 2A, lane 8) and in Southern blot analysis (Fig. 2B, lane 1).

One mouse (6) died shortly after birth and the remaining five were used as founders (F_0) for further breeding. Two of the founders (mice 37 and 54) were unsuccessful in reproduction.

Selection of transgenic offsprings (F_1, F_2 . . .) were based upon further PCR analysis (data not shown).

hPTH mRNA Analysis by PCR and Southern Blot in Tissue From Transgenic Mice

Of a total of nine individuals examined, two F_2 females from breeding lines 33 and 47 were selected for demonstration of tissue specific expression of the transgene by hPTH mRNA PCR analysis. hPTH mRNA was detected exclusively in the mammary gland in both lines (Fig. 3, lanes 3 and 4). The hPTH mRNA positive tissues from both animals had a correct PCR amplified DNA band (Fig. 3) compatible with the specific primers *p4* and *p5* used for PCR from reverse transcribed mRNA (Fig. 1 and see Materials and Methods). There were no detectable hPTH mRNA in liver, salivary gland or heart (Fig. 3, lanes 5, 6, and 7), nor in brain, lungs, or kidneys (data not shown) from these or other transgenic mice. Mammary gland from non-transgenic control mice were negative for hPTH transcripts (Fig. 3, lane 2). tRNA (2 μg) added to hPTH mRNA included as a positive control resulted in the expected PCR amplified DNA band (Fig. 3, lane 8).

Fig. 3. PCR analysis of total RNA extracted from selected tissues of transgenic mice from F_2. *Lanes 1* and *9:* Molecular size markers as in Figure 2A. *Lane 2:* Mammary gland from non-injected, control mouse. *Lane 3:* Mammary gland, mouse 33. *Lane 4:* Mammary gland, mouse 47. *Lane 5:* Liver, mouse 47. *Lane 6:* submandibular gland, mouse 47. *Lane 7:* Heart, mouse 47. *Lane 8:* 2 μg tRNA added to hPTH mRNA.

For improved sensitivity the samples in Figure 3 were analyzed by Southern blot using the WAPPTHSV2 as hybridization probe, and the results were in concordance with those from the hPTH mRNA PCR analysis (data not shown).

Chemiluminometric Immunoassay of Intact hPTH in Mouse Milk

Samples of milk, diluted 1:50 with distilled water, collected the fifth and tenth day post partum from nine individuals in F_1 and eight in F_2 from three different breeding lines (32, 33, and 47) were analyzed by two-site chemiluminometric immunoassay for the presence of intact hPTH (1-84). Fifteen samples (from breeding lines 32, 33, and 47) of the 17 samples analyzed contained intact hPTH in the range 10–415 ng/ml milk (Table I). Two samples (from breeding line 32) contained trace levels of hPTH, but below the defined lower detection limit (see Materials and Methods) (Table I). Because of reduced fertility of F_0 37 and F_0 54 it was not possible to collect enough milk for hPTH measurements. Milk from ten non-transgenic individuals analyzed several times as negative controls contained no detectable hPTH.

Chemiluminometric Immunoassay of Intact hPTH in Mouse Blood

The blood sampling from mice invariably resulted in immediate haemolysis. In order to avoid the interference by haemoglobin with the measurements which could result in uncorrectly low values for the hPTH concentrations, part purification of hPTH from the plasma had to be carried out. Samples collected the tenth day post partum from the two individuals (mice 33-6 and 47-6 in F_2), demonstrating the highest levels of hPTH concentrations in milk, and from ten non-transgenic controls (Table I), were analyzed by two-site chemiluminometric immunoassay for the presence of intact hPTH (1-84). The concentrations of hPTH were 535 ng/ml plasma for mouse 33-6 and 686 ng/ml plasma for mouse 47-6 (Table I). The average value of PTH measured in samples from the non-transgenic individuals analyzed several times as negative con-

TABLE I. Concentrations of hPTH in Milk and Plasma From Transgenic Mice*

Mouse no.	Generation	Milk		Plasma
		5 days post-partum	10 days post-partum	10 days post-partum
32-5	F_1	9	10	—
32-6	F_2	—	13	—
32-7	F_2	n.d.	n.d.	—
33-4	F_1	163	268	—
33-5	F_2	273	248	—
33-6	F_2	180	313	535
47-5	F_1	288	403	—
47-4	F_2	110	198	—
47-6	F_2	243	415	686
Non-transgenic (average value) (n = 10)		0	0	352 (310–467)

*All figures are given in ng hPTH/ml. — = no samples were collected for analysis. n.d. = Not detectable.

trols was 352 ng/ml (range 310–467 ng/ml; n = 10), indicating a crossreactivity between mouse and human PTH and slightly raised levels of circulating hPTH in the transgenic mice.

Analysis of Purified hPTH From Milk by Polyacrylamide Gel Electrophoresis and Immunoblotting

Mouse milk from selected individuals (33 and 47) was collected for further characterization of the hPTH immunoreactive material. After dilution, centrifugation, acidification, and reversed phase HPLC (see Materials and Methods) a peak appeared comigrating with the hPTH standard (data not shown). These fractions were pooled and subjected to analysis by SDS-PAGE followed by immunoblotting, and one hPTH peptide band of correct electrophoretic mobility (9.4 kD) was demonstrated (Fig. 4, lane 3). No smaller hPTH fragments could be detected by analysis of this and other HPLC fractions (data not shown).

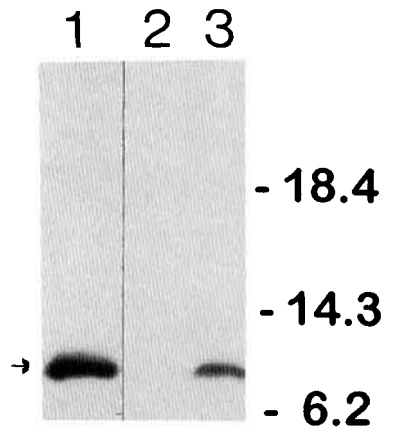

Fig. 4. Characterization by SDS-PAGE followed by immunoblot analysis of purified hPTH extracted from 2 ml milk of transgenic mice. A C-terminal (44–68 amino acid region) specific antiserum was used [Gautvik et al., 1979]. *Lane 1:* 1 μg hPTH standard as a recombinant peptide produced in yeast, 9.4 kD, indicated by horizontal arrow. *Lane 2:* Extract from non-transgenic control mouse. *Lane 3:* Extract (0.8 μg hPTH as quantitated by immunoassay before the extraction procedure) from the breeding line of mouse 47. Exposure time was 8 h.

DISCUSSION

To our knowledge this is the first report to describe a true transgenic animal expressing hPTH, and in a tissue specific manner. However, uncharacterized PTH related material has been expressed in non-transgenic rats by fibroblasts infected with retrovirus encoding hPTH and implanted intraperitoneally [Nussbaum et al., 1989]. Expression of hPTH in insect cells and silkworm larvae transfected by a recombinant baculovirus vector and in transfected mammalian cells in culture, resulted in an intact hormone showing that a eukaryotic expression system is capable of making a stable and authentic peptide [Rokkones et al., 1994; Mathavan et al., 1994]. However, the high cost of tissue cell cultures eliminates this as a possible future production system.

Regulatory sequences from WAP fused to different coding genes are reported to direct the expression of peptides in varying amounts (range 0.2–410 mg/l mouse milk) [Yu et al., 1989; Pittius et al., 1988; Reddy et al., 1991; Gunzburg et al., 1991], and our results fall into the lower part of this range (Table I). Experiments with transgenic sheep expressing the human blood clotting factor IX or human α1-antitrypsin (hα-1AT) in milk show that this expression system can be

applied to dairy livestock [Clark et al., 1989; Wright et al., 1991].

An inherent problem in making a transgenic animal expressing hPTH is the possible unregulated secretion of the hormone to the circulation, probably creating a hypercalcemia and serious skeletal derangement. Our measurements of hPTH in the blood from transgenic mice may suggest a small to moderate "leakage" of hPTH from the secretory cells of the mammary gland into the blood as indicated by an increase of measured hPTH levels in plasma of about 50–100% above basic level (Table I). It is possible, but hardly likely, that "hyperparathyroidism" can explain why one of our transgenic mice died shortly after birth. However, a recent report [Devinoy et al., 1994] describes the presence of hGH in the blood and possible systemic effects of hGH in transgenic mice harbouring a WAPhGH construct expressed in the mammary gland. Thus, an hPTH-transgenic animal ought to express and secrete the hormone through an exocrine gland or the kidneys in order to avoid systemic effects. The kidney as a target organ, but not the mammary gland, may be expected to degrade the hormone leading to low yield and/or spurious hPTH induced effects.

The measurements of hPTH in the plasma of non-transgenic controls (Table I) resulting in an average value of 352 ng/ml, indicate a reaction between the two anti-hPTH antibodies used and mouse PTH. The amino acid sequence of mouse PTH is not published, but a comparison of the amino acid sequences of rat PTH [Heinrich et al., 1984; Schmelzer et al., 1987] and human PTH revealed an overall homology of 89%. The regions recognized by the antibodies, the N-terminal (1–34), and the mid-region (44–68) showed a homology of 94 and 88%, respectively. Assuming that rat and mouse PTH are highly homologous, a cross-reaction between the anti-hPTH antibodies and mouse PTH is likely to occur.

The frequency of transgenesis was about 10% (6/58 of the offspring), somewhat less than expected [Palmiter and Brinster, 1985], and three lines of transgenic mice were established. Selection of founder mice was based upon compatible results in F_0 from PCR and Southern blot analyses of tail tissue using primers specific for WAP-hPTH sequences resulting in a band of expected size (Figs. 1 and 2A), and a probe hybridizing with SV40 sequences, respectively (Fig. 1), indicating no false positives (Fig. 2A and B). BglII digest of genomic DNA from six potential transgenic mice analyzed by Southern blot revealed bands of about 6 kb (varying in size from 5.6–6.6 kb in the six individuals as exemplified by mouse 47 in Fig. 2B, lane 2) indicating that the 3,25 kb injected gene construct (WAPPTHSV2) was integrated as a single copy presenting the unique BglII restriction cleavage site inside the construct (Fig. 2A, lane 2).

The PCR analysis, confirmed by Southern blot analysis, of cDNA synthesized from total RNA extracts from different tissues in selected transgenic mice (33 and 47) showed that the transgene was expressed in the mammary gland only (Fig. 3). We could not confirm expression of the transgene in extramammary tissues as reported by others [Pittius et al., 1988] nor in non-transgenics. The concentration of hPTH mRNA in the mammary gland was too low to be detected by conventional Northern blot analysis.

The 2.5 kb 5′ regulatory sequences of WAP terminating downstream of the transcription start site used in the WAPPTHSV2 construct have also been used in transgenic mice to direct tissue specific and hormone dependent expression of the two oncogenes human Ha-ras [Andres et al., 1987, 1988] and murine c-myc [Andres et al., 1988] and human tissue plasminogen activator (hTPA) [Pittius et al., 1988]. This fragment contains binding sites for proteins that are specific to lactating mammary and which may be cis-acting regulatory elements [Lubon and Hennighausen, 1987]. Like in our study the levels of RNA of the two oncogenes were very low and estimated to be appreciably lower than that of the endogenous WAP gene.

We have used the hPTH cDNA in our experiments and not the genomic DNA. Some investigators claim [Wilmut et al., 1991] that introns and flanking sequences in the genomic gene are necessary for regulated expression of the transgene as exemplified by the large amount of α1-AT produced in milk when genomic DNA was used (6–7 mg/ml) compared to experiments with the α1-AT cDNA (5 μg/ml) both using the β-LAC regulatory sequences [Archibald et al., 1990]. However, a WAP construct expressing the breast cancer protein PS2 gave no detectable levels in milk using the genomic DNA, but a concentration of 40 mg/l milk with the PS2 cDNA [Tomasetto et al., 1989]. Therefore, general rules about the expression efficiency of a certain gene construct are difficult to define.

It remains to be shown whether the relatively constant level of hPTH expressed in mouse milk

throughout the lactation period (Table I) could be increased from the moderate concentrations in this report (up to 415 ng hPTH/ml milk) by using the hPTH gene with, e.g., the entire WAP gene and flanking sequences as the rest of the expression cassette.

Two milk samples from mouse 32-7 contained only traces of hPTH below the detection level of immunoassay (Table I). The mouse was transgenic according to the PCR analysis (data not shown), but the transgene may have been inserted into a transcriptionally inactive region of the genome. However, this was not analyzed in further detail.

The milking pump of our own design easily collected 1–2 ml milk from each mouse and did not impair the subsequent lactation of the offspring. The concentrations of hPTH in milk (Table I) measured by two-site chemiluminometric immunoassay were compatible with the results from SDS-PAGE and immunoblotting (Fig. 4). No fragmentation of this protease sensitive hormone could be demonstrated in milk (Fig. 4) as previously reported in other systems [Rabbani et al., 1988; Høgset et al., 1990b; Gabrielsen et al., 1990].

The hPTH recovered from the milk had the same electrophoretic mobility (9.4 kD) and immunologic properties in immunoblots from SDS-PAGE as standard hPTH (Fig. 4), indicating that the hPTH produced in the mammary gland is very similar or identical to native hPTH.

This report demonstrates that it is possible to produce intact hPTH in the mammary gland of transgenic mice. The potential advantages are the capability to produce therapeutic important human peptide hormones in large quantities in a highly cost effective system without detriment to the producing animal.

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