Epidermal growth factor-stimulated parathyroid hormone-related protein expression involves increased gene transcription and mRNA stability

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Epidermal growth factor (EGF) produced rapid and striking effects on parathyroid hormone-related protein (PTHrP) gene expression in the immortalized human keratinocyte cell line, HaCaT. Steady-state levels of PTHrP mRNA and secreted PTHrP were increased 10-fold by maximally effective concentrations of EGF. EGF increased both PTHrP gene transcription and PTHrP mRNA stability. Nuclear run-on assays demonstrated a 4-fold increase in transcriptional rate in EGF-stimulated cells while transient transfection analysis indicated that the action of EGF on transcription involved both the GC-rich promoter, P2, and the downstream TATA promoter, P3, but apparently not the upstream TATA promoter, P1. In experiments where EGF treatment produced more stable PTHrP transcripts,

the half-life of *c-fos* mRNA was unaltered, suggesting a relatively specific effect of EGF. Moreover, only those species of PTHrP mRNA containing two of the alternative 3' exons (exons VII and VIII) were stable, those containing exon IX were not. Reverse-transcription PCR demonstrated that EGF produced differential increases in the abundance of PTHrP mRNA species initiated by the three PTHrP promoters. The major effect was seen on the abundance of transcripts initiated by P1 and P2, with less marked regulation of P3-initiated transcripts. Thus EGF regulation of PTHrP gene expression in HaCaT cells is multifactorial and the combination of its actions at the 5' and 3' ends of the gene favours the accumulation of subpopulations of PTHrP mRNA containing exons I, VII and VIII.

INTRODUCTION

The hypercalcaemic protein, parathyroid hormone-related protein (PTHrP) shares N-terminal sequence similarity with parathyroid hormone (PTH) [1], binds to the same receptor [2] and produces classical PTH-like effects in tissues such as bone and kidney [3–5]. PTHrP is overexpressed in squamous carcinoma and by several other types of tumour and circulates at elevated concentrations in patients with humoral hypercalcaemia of malignancy [6,7].

The human PTHrP gene is complex: nine exons are present, with coding sequences located only in exons V, VI, VIII and IX (Figure 1). Alternative usage of exons VII, VIII and IX generates transcripts encoding three isoforms of PTHrP of 139, 173 and 141 amino acids in length respectively. The relatively simple PTH gene shares similar exon–intron organization, suggesting that the two are derived from the same ancestral gene (for reviews see [8,9]). Three functional promoters have been characterized in the human PTHrP gene: P1 and P3 are classical canonical TATA promoters and initiate transcription at exons I and IV respectively [10–13], while P2 is high in GC content and initiates transcription 11 nucleotides 5' to the splice acceptor site of exon III [14]. There is potential for alternative splicing at both the 5' and the 3' ends of the primary human PTHrP transcripts. Although these characteristics allow for the production of at least 15 mature mRNA species (Figure 1), the relative abundance of these has not been determined. The pattern is likely to be highly tissue-specific and responsive to external factors, such as hormones and

growth factors, all acting in concert to regulate the frequency of promoter usage and specific splicing events.

Although PTHrP was first identified as a tumour product, its specific distribution in various normal tissues, including skin [15,16], placenta [17], uterus [18,19], smooth muscle [20,21] and lactating mammary gland [22] as well as in many tissues of the human fetus [23] indicate an important role for PTHrP in normal physiology. Mice homozygous for a null mutation in the PTHrP

Figure 1 Schematic representation of the human PTHrP gene

The PTHrP gene comprises nine exons which are transcribed from three promoters designated P1, P2 and P3. Exons encoding UTRs are shown as open boxes, while exons encoding coding regions are stippled boxes. The observed patterns of alternative splicing are shown as solid lines below the gene. Thus transcripts originating from the activity of P1 always contain exon I but this exon may be spliced directly to either exon II, III or V in mature transcripts [43]. Coupled to differential usage of the three 3' exons this generates nine mRNA products from P1 alone. Three mature transcripts may originate from P2; all contain exons III, V and VI, plus either exon VII, VIII or IX. Similarly P3-containing transcripts all contain exons IV, V and VI plus either exon VII, VIII or IX. Together the 15 mature transcripts generate a broad hybridization signal on Northern blots. Three protein isoforms are encoded; these comprise 139, 173 or 141 amino acid residues and derive from mRNA species containing exons VII, VIII or IX respectively.

Abbreviations used: ALP, alkaline phosphatase; CAT, chloramphenicol acetyltransferase; DRB, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGF-R, EGF receptor; FBS, fetal bovine serum; GAPDH, glyceraldehyde phosphate dehydrogenase; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein; RT-PCR, reverse-transcription PCR; SV-40, simian virus 40; UTR, untranslated region.

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gene show abnormal skeletal development and die soon after birth [24]. The production of a PTH-like substance by normal keratinocytes was first observed by Merendino et al. in 1986 [25], and since then others have shown that primary human keratinocytes and immortalized and transformed keratinocyte cell lines produce PTHrP in culture [26–29]. However, the function of PTHrP in this tissue is unknown. When HPV16-immortalized human keratinocytes were stably transfected with a vector encoding antisense PTHrP (1–141) RNA, the inactivation of PTHrP allowed the cells to grow more rapidly [30]. Conversely, epidermal growth factor (EGF), a potent stimulator of keratinocyte growth, stimulates the production of PTHrP in normal human and rat keratinocytes [25,27] and also in the human osteosarcoma-derived cell line, SaOs-2/B10 [31].

To study the mechanisms involved in EGF-stimulated PTHrP production by keratinocytes, we utilized the immortalized human keratinocyte cell line, HaCaT [32], which arose spontaneously in long-term culture of adult skin. The immortal character of the cells is probably conferred, at least in part, by the presence of point mutations on both alleles of the p53 gene [33]. In transplantation experiments *in vivo* HaCaT cells expressed the normal array of keratin types and produced a fully ordered epidermis [32]. We found that HaCaT cells produced PTHrP constitutively, the levels of which could be dramatically stimulated by treatment with nanomolar concentrations of EGF. The HaCaT cell line therefore provided a suitable model in which to study the mechanisms underlying EGF-stimulated PTHrP gene expression.

EXPERIMENTAL

Cell cultures

The clonal cell line, HaCaT, originated in a population of cells grown out from a long-term culture of normal human skin [32]. The cells were routinely maintained in Dulbecco's modification of Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) and subcultured (1:20) every 6–7 days using 0.5 mM EDTA in PBS (15 min at 37 °C) followed by a further 15 min in 0.025% trypsin/0.5 mM EDTA. RNA was prepared from confluent cells in 150-cm² Petri dishes incubated in DMEM containing 0.1% BSA for 16–24 h prior to EGF stimulation. For experiments to determine the half-life of PTHrP mRNA, confluent cells were either untreated or stimulated with EGF for 2 h, and then exposed to actinomycin D (0.8 µM) or the specific inhibitor of RNA polymerase II, DRB (5,6-dichloro-1-β-D ribofuranosylbenzimidazole; Sigma-Aldrich Co., St. Louis, MO, U.S.A.) at a concentration of 25 µg/ml and RNA extracted from duplicate cultures at timed intervals thereafter.

Assays of PTHrP

HaCaT cells were subcultured (1:20) into the wells (area 9.6 cm²) of 6-well plates and grown to confluence (8 × 10⁵ cells) in DMEM containing 10% FBS. The cells were then rinsed with serum-free DMEM and cultured for 24 h in DMEM containing 0.1% BSA (2 ml) in the presence or absence of EGF. PTHrP concentrations in culture supernatants from HaCaT cells were determined in an N-terminal radioimmunoassay utilizing a polyclonal goat antiserum against the first 40 amino acids of the PTHrP molecule [PTHrP (1–40)] with a sensitivity of 2 pM [7]. Recombinant PTHrP (1–84) used as standard was diluted in blank culture medium (DMEM containing 0.1% BSA) to correct for possible non-specific protein interference with antibody binding to tracer. Samples were assayed in a range of dilutions, demonstrating parallelism to the standard curve. Biologically active PTHrP was assayed in the same supernatants using the PTHrP/PTH-re-

sponsive osteosarcoma-derived cell line, UMR 106.01, with measurements of cyclic AMP accumulation as previously described [34]. Synthetic PTHrP (1–34) demonstrates the full biological activity of the intact PTHrP molecule in this assay and was used as standard.

Northern-blot analysis

RNA was extracted from HaCaT cells using the method of Chomczynski and Sacchi [35] and separated on 1% (w/v) agarose gels containing formaldehyde. RNA was transferred overnight by capillary action [36] to nylon filters (Hybond N, Amersham International, Bucks., U.K.) and immobilized by exposure to UV light. Filters were pre-hybridized in a buffer containing 50% deionized formamide, 5 × Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA), 5 × SSPE (1 × SSPE = 0.15 M NaCl, 0.01 M NaH₂PO₄·2H₂O, 1.2 mM EDTA, pH 7.4), 0.5% SDS and 1% (w/v) skimmed milk powder for at least 6 h at 42 °C. The filters were then probed in fresh hybridization solution with the full-length cDNA insert from pBRF61 [1] encompassing human PTHrP exons III, V, VI and IX, or probes specific for individual exons, as follows. The exon VII probe was the 480 bp *Hind*III–*Eco*RI fragment of the human PTHrP cDNA clone 10B5 [37], and probes specific for exons VIII or IX were generated by amplification of human genomic DNA using primers complementary to the 5' and 3' ends of each exon with *Bam*HI restriction sites to facilitate cloning. All probes were labelled with [α -³²P]dCTP (1800 Ci/mmol; Amersham International) using either nick-translation or random-primer kits (Boehringer-Mannheim Biochemica, Mannheim, Germany). The signals were visualized by autoradiography and quantified using a Molecular Dynamics PhosphorImager. Subsequently, hybridized cDNA was removed from the filters by washing in a solution containing 5 mM Tris, pH 7.4, 2 mM EDTA and 0.1 × Denhardt's solution at 65 °C for 1–2 h. Some filters were re-probed with the cDNA encoding the human transcription factor *c-fos* [38]. To permit quantification of the mRNA signals, the filters were then re-probed with either ³²P-labelled chicken glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA [39] or an oligonucleotide specific for 18 S rRNA [40] labelled with [γ -³²P]dATP using T4 polynucleotide kinase.

Nuclear run-on assay

Near-confluent monolayers of HaCaT cells were incubated with EGF (10 nM) for timed intervals between 0 h and 8 h. Isolation of nuclei, *in vitro* transcription and hybridization were carried out essentially as described by McKnight and Palmiter [41] with minor modifications [42]. For each sample, approximately (12–15) × 10⁶ nuclei were isolated by gentle homogenization of HaCaT cells on ice in hypotonic buffer, retaining the cytoplasmic fraction for extraction of cytoplasmic RNA. Isolated nuclei were incubated for 20 min at 25 °C in a transcription mixture containing 100 µCi of [α -³²P]UTP (800 Ci/mmol), followed by digestion with DNase I and Proteinase K [42]. RNA was extracted with phenol/chloroform and precipitated with ethanol. The pellets were washed with 70% ethanol and re-dissolved in 0.3 ml of solution D [4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% (v/v) Sarkosyl] and re-precipitated in ethanol for 2 h at –20 °C. The pellets were washed with 80% ethanol, dried and resuspended in 10 mM Tris/HCl, pH 7.4, 1 mM EDTA. The ³²P-labelled transcripts (6 × 10⁶ c.p.m./sample) were incubated with strips of nylon-supported nitrocellulose filters (Hybond C-extra, Amersham International) on to which the following cDNAs had been previously immobilized using a Bio-Rad Bio-Dot microfiltration apparatus: (i) the 1.2 kb insert of PTHrP

cDNA of pBRF61 (1 μ g; [1]), (ii) the 1.2 kb insert of chicken GAPDH cDNA (1 μ g), (iii) the linearized plasmid, pBRF61 (10 μ g) and (iv) the linearized vector control, pUC119 (10 μ g; [36]). Prehybridization was for 16 h at 42 °C in a solution containing 50 % formamide, 5 $\times$ SSC (1 $\times$ SSC = 0.15 M NaCl, 0.015 M sodium citrate), 5 $\times$ Denhardt's solution, 10 mM EDTA, 100 mM Tris/HCl, pH 7.4, 20 μ g/ml tRNA, 0.1 % SDS and 100 μ g/ml sonicated salmon sperm DNA. Hybridization was for 72 h at 42 °C in the same buffer (1 ml/sample); filters were then washed three times at room temperature in 2 $\times$ SSC for 5 min and twice at 65 °C in 0.1 $\times$ SSC, 0.1 % SDS for 20 min. Hybridization signals were visualized by autoradiography after 5 days at -70 °C with intensifying screens. The relative intensity of signals was measured with a Molecular Dynamics Phosphor-Imager which enabled accurate quantification of weak signals.

Transient transfection experiments

To examine the effect of EGF on the promoter activity of the human PTHrP gene, HaCaT cells were transfected with constructs containing fragments of the 5' flanking sequence of the PTHrP gene ligated to a promoterless bacterial chloramphenicol acetyltransferase (CAT) gene. For pSMR492, the P1-harboring plasmid pSMR218 [43] containing the *Bgl*II fragment of the PTHrP gene (-3589 to -2507 bp from the initiating ATG) was digested with *Hind*III and the fragment encompassing the 3' sequences of exon I, all of intron I and the simian virus 40 (SV-40) promoter region of pCAT promoter was removed. The resulting plasmid contained 433 bp of the PTHrP gene from the *Bgl*II site at -3589 to the *Hind*III site at -3156 (see Figure 5a), corresponding to 355 bp 5' of the P1-transcription start site and 78 bp of exon I. pSMR38 contains 1100 bp of PTHrP DNA including exons III and IV and the 5' end of exon V, 20 bp short of the ATG translation start site [44]. pZMR30 contains a truncated fragment of this region with a 5' terminus at position -530 relative to the ATG, cloned into pCATbasic (Promega Corporation, Madison, WI, U.S.A.) [45]. Constructs were verified by DNA sequencing. Transfection of HaCaT cells was carried out 24 h after seeding 0.5×10^6 cells into 9.6-cm² dishes. Unless stated otherwise, the cultures were transfected with 5 μ g of circular plasmid DNA for 4 h using the calcium phosphate coprecipitation method of Gorman et al. [46] with the addition of 50 μ g/ml chloroquine. Following glycerol shock (15 % glycerol in serum-free DMEM) for 90 s, cells were rinsed three times in DMEM and incubated for a further 48 h in DMEM containing 10 % charcoal-stripped FBS with EGF or vehicle. To determine the transfection efficiency of each sample, cells were co-transfected with 2.5 μ g of the reporter plasmid, pSV₂Apap, containing the placental alkaline phosphatase (ALP) gene driven by the SV-40 early promoter [47]. Placental ALP was assayed in cell extracts by standard spectrophotometric procedures as described previously [42]. Cell extracts were assayed for CAT activity according to published procedures [46] and this was expressed as percentage conversion of [¹⁴C]chloramphenicol to acetylated forms for samples containing equivalent placental ALP activity.

Reverse-transcription PCR

For reverse-transcription PCR (RT-PCR), 5 μ g of total RNA was reverse transcribed using AMV reverse transcriptase (Promega) according to the manufacturer's instructions and using oligo(dT)₁₅ primer (Promega). One twenty-fifth of the reaction was used per PCR in a reaction mixture containing 50 pmol of each primer, 25 μ M each of dATP, dGTP, dTTP and dCTP (Pharmacia, Uppsala, Sweden), 2 μ l of 10 $\times$ reaction buffer,

1 unit of *Taq* DNA polymerase (Boehringer Mannheim) and sterile distilled water to a total volume of 20 μ l. The mixture was overlaid with 50 μ l of paraffin oil and amplification was performed in a Perkin-Elmer DNA thermal cycler 480 programmed for denaturation (92 °C for 30 s), annealing (55 °C for 30 s) and extension (72 °C for 1 min) as follows: 20 cycles for (GAPDH-3-GAPDH-4), 22 cycles (obrf 15.83-obrf 15.89, obrf 15.84-obrf 15.89), and 24 cycles (obrf 15.93-15.89, obrf 15.95-obrf 15.89). We verified that under these conditions, the yield of amplified product from all the primer combinations (see Figure 5a) was directly proportional to the number of PCR cycles and amount of input RT-RNA (data not shown). The PCR products were resolved in a 2 % (w/v) agarose gel and identified by Southern transfer followed by hybridization with a ³²P-labelled internal oligonucleotide probe, obrf 15.8. The target sequence for this probe, as well as the positions for binding of the PCR primers, are denoted by the horizontal arrows in Figure 5(a). All oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer model 381A and have been described previously [43].

RESULTS

Using an N-terminal PTHrP RIA [7], we found that confluent cultures of HaCaT cells constitutively produced significant levels of PTHrP, 300 pmol/l, over a 24 h period. These levels were greatly stimulated when EGF was added to the medium (Figure 2). The effect was concentration-dependent with an ED₅₀ of approximately 2 nM EGF. At the maximal concentration of 10 nM, EGF stimulated PTHrP production by approximately 10-fold. Most of the PTHrP secreted into the medium was found to be biologically active in a bioassay based on the ability of PTHrP to stimulate cyclic AMP accumulation in the osteogenic osteosarcoma-derived cell line, UMR 106.01 [34]. Figure 2 demonstrates that the levels of bioactive PTHrP were concordant with those of immunoreactive PTHrP.

EGF (1 nM) stimulated PTHrP mRNA levels within 0.5 h of treatment (Figure 3a). The complexity of the hybridization signal

Figure 2 Production of immunoreactive PTHrP and biologically active PTHrP by HaCaT cells in response to EGF

HaCaT cells were grown to confluence in 9.6-cm² wells in DMEM containing 10 % FBS. The medium was then replaced with 2 ml of DMEM containing 0.1 % BSA and EGF at the concentrations indicated. The medium was collected after 24 h and 100 μ l aliquots taken for measurement of immunoreactive PTHrP (○) and biologically active PTHrP (●) as described in the Experimental section. Each point represents the mean of triplicate determinations $\pm$ S.E.M.

Figure 3 Northern-blot analysis of the effect of EGF and cycloheximide on PTHrP mRNA

HaCaT cells were grown to confluence in 150-cm² Petri dishes in DMEM containing 10% FBS. The cells were then rinsed in serum-free DMEM and incubated for 16 h in DMEM containing 0.1% BSA prior to the addition of EGF in the presence or absence of 35 μ M cycloheximide as indicated. At the end of the experiment the cells were lysed in solution D [35] and total cytoplasmic RNA prepared for Northern-blot analysis as described in the Experimental section. (a) Time-dependent effect of addition of 1 nM EGF. HaCaT cells were exposed to 1 nM EGF for 0.5 h, 1 h, 2 h, 4 h, 8 h, 12 h, 14 h, 16 h and 24 h as indicated and the levels of PTHrP mRNA compared with those of control cells (time = 0 h). (b) Effect of cycloheximide. In the same experiment, HaCaT cells were treated simultaneously with 1 nM EGF and 35 μ M cycloheximide (CHX) for the times indicated. After autoradiography to visualize PTHrP mRNA levels, the filters were stripped and re-probed with a cDNA corresponding to chicken GAPDH [39] to permit quantification of the hybridization signals. (c) The quantification is expressed graphically as the treated/control ratio, which was equal to 1 at $t = 0$ h.

was characteristic of the presence of multiple PTHrP mRNA species produced by alternative splicing events at the 5' and 3' ends of the primary transcripts. The initially rapid increase in the abundance of PTHrP mRNA was followed by a relatively slow rise to the new steady-state level, which was about 8–10-fold elevated over controls. Maximal levels of PTHrP mRNA persisted for 10–16 h but were diminishing by 24 h (Figure 3a). A concentration-dependent effect of EGF on PTHrP expression was also observed at the mRNA level; a maximal effect was obtained after treatment with 1 nM EGF (data not shown). In the experiment depicted in Figure 3(b), treatment with the protein synthesis inhibitor, cycloheximide (35 μ M), did not markedly affect the production of PTHrP mRNA by HaCaT cells, either alone or in combination with EGF, indicating that the action of EGF was independent of ongoing protein synthesis.

Figure 4 Nuclear run-on analysis of PTHrP transcription rate

(a) Radiolabelled transcripts were prepared from the nuclei of untreated (control) HaCaT cells or cells treated with 10 nM EGF for 0.5 h, 1 h, 2 h, and 8 h, as described in the Experimental section. The transcripts were then allowed to hybridize to immobilized cDNAs on strips of nylon-supported nitrocellulose filters for 48 h, prior to washing and visualization of hybridization signals by autoradiography. The immobilized cDNAs were as follows: PTHrP, the 1.2 kb insert from pBRF61 (1 μ g/slot; [1]) encoding exons III, V, VI and IX of the PTHrP gene; GAPDH, a 1.2 kb insert of chicken GAPDH cDNA (1 μ g/slot); pBRF61, the linearized plasmid pBRF61 (10 μ g/slot); pUC119, the linearized plasmid pUC119 (10 μ g/slot). The relative intensity of the PTHrP signal to the GAPDH signal was quantified using a Molecular Dynamics PhosphorImager and ImageQuant software. The control ratio was set at 1.0, and the change in relative intensity indicated below the appropriate filter. (b) Concurrent accumulation of PTHrP mRNA in the cytoplasm of the HaCaT cells in which the nuclear run-on analysis (a) was conducted. The increase in abundance of the PTHrP mRNA signal in response to EGF was calculated by reference to the 18 S rRNA signal.

This contrasts with previous studies with a variety of PTHrP-expressing cells, including rat keratinocytes, where treatment with cycloheximide strongly stimulated PTHrP mRNA levels [28,48] as well as augmenting the response produced by transforming growth factor β (TGF β) [44].

The effect of EGF on PTHrP gene transcription was determined in nuclear run-on experiments. EGF treatment (0.5 h) increased the abundance of radiolabelled nuclear PTHrP transcripts approximately 4-fold relative to unstimulated controls (Figure 4a). This increased rate of transcription was maintained for 8 h and was accompanied by a 10-fold increase in the abundance of cytoplasmic PTHrP mRNA (Figure 4b). This result demonstrated that the EGF-stimulated increase in PTHrP mRNA in HaCaT cells was produced, at least in part, by increased transcriptional activity of the PTHrP gene.

To study further the transcriptional effect of EGF on expression of the PTHrP gene, HaCaT cells were transiently transfected with expression vectors containing fragments of the

Figure 5 5' flanking region of the PTHrP gene and transient transfection analysis

(a) Schematic representation of the 5' region of the human PTHrP gene showing the three promoters, P1, P2 and P3. The oligonucleotide primers, used in the RT-PCR analysis shown in Figure 8, are represented by horizontal arrows above and below the gene. The (–3589 to –3156) fragment of the P1 promoter contained in pSMR492 is shown directly below the relevant portion of the gene and the expanded region shows the *Bam*HI–*Xho*I fragment used to generate the PTHrP-CAT expression vectors, pSMR38 and pZMR30. The black box in exon III represents the 11 bp sequence, immediately upstream of the splice acceptor site in exon III, which is unique to P2-initiated transcripts. (b) Concentration-dependent increase in CAT activity in lysates of HaCaT cells co-transfected with pSMR38 and pSV₂pap (see the Experimental section) and treated with EGF, as indicated. CAT activity was determined using standard procedures for samples containing equal amounts of placental ALP activity and expressed as the percentage conversion of [¹⁴C]chloramphenicol into acetylated forms. Each value represents the mean of triplicate determinations ± S.E.M. (c) Comparison of the effect of EGF on CAT activity in lysates of HaCaT cells co-transfected with pSMR492, pSMR38 or pZMR30 and pSV₂pap, and treated in the presence (EGF) or absence (CON) of 5 nM EGF. Only very weak activity is associated with pSMR492; this panel depicts the CAT activity associated with cell lysates transfected with 10 µg of pSMR492 (compared with 3.5 µg of both pSMR38 and pZMR30) and assayed after a 6 h incubation period (compared with 3 h for pSMR38 and pZMR30). Each value represents the mean of three samples (of which two representatives are shown) ± S.E.M. This experiment was conducted four times with similar results.

5' flanking region of the PTHrP gene fused to the bacterial CAT gene (Figure 5). A clear concentration-dependent effect of EGF was observed on the expression of CAT activity by HaCaT cells transfected with a plasmid, pSMR38 [44], containing the 5'-flanking sequence –1118 to –20 from the initiation of translation codon, ATG (Figures 5a and 5b). This region includes both the GC-rich (P2) and the downstream TATA (P3) promoters. There was a 6-fold increase in CAT activity in response

Figure 6 Stability of PTHrP mRNA in the presence of EGF

RNA was prepared from confluent HaCaT cells treated with the transcriptional inhibitor, actinomycin D, as described in the Experimental section. The left-hand portion of the autoradiograph (upper panel) shows the abundance of PTHrP mRNA in HaCaT cells treated with 1 nM EGF for 2 h prior to the addition of actinomycin D (0.8 µM) for the times indicated. The right-hand side of the autoradiograph shows the relatively rapid decay of PTHrP mRNA from HaCaT cells treated with actinomycin D during the same time-course but in the absence of prior stimulation with EGF. The relative intensity of the PTHrP mRNA signal in each lane was quantified using a PhosphorImager and expressed as a percentage of the signal obtained at the time of actinomycin D addition ($t = 0$ h). The filter was subsequently re-probed with human *c-fos* and chicken GAPDH cDNAs to demonstrate the effect of these treatments on other mRNA species. The rate of decay of PTHrP mRNA in the presence and absence of EGF is expressed graphically in the lower panel, left-hand side, and that of *c-fos* mRNA on the right-hand side. Hybridization with the 18 S oligonucleotide demonstrated closely comparable levels of 18 S RNA in all lanes (data not shown).

to 10 nM EGF (Figure 5b). Figure 4(c) depicts the CAT activity in extracts of HaCaT cells transfected with pSMR492, pSMR38 and pZMR30 [45]. pSMR492 contains the upstream TATA promoter (P1) and 355 bp of 5' flanking sequence, while pZMR30 contains the sequence –530 to –20, thereby excluding the GC-rich promoter, P2 [14]. The effect of EGF on the CAT activity of cells transfected with both these constructs was consistently less marked than that produced by cells transfected with pSMR38 (Figure 5c). Indeed the P1-containing construct had only very weak basal activity and this was not stimulated at all by EGF. The diminished effect of EGF on pZMR30 compared with pSMR38 may indicate that P3 was stimulated less by EGF than P2; however, the decreased responsiveness of pZMR30 may also result from the deletion of *cis*-acting sequences present in the region –1118 to –530 which may confer EGF-regulation upon P3 transcription.

The half-life of PTHrP mRNA was investigated in HaCaT cells using two transcriptional inhibitors: actinomycin D, which blocks all RNA synthesis, and DRB, a specific inhibitor of RNA polymerase II, which selectively blocks mRNA synthesis. The half-life of PTHrP mRNA in unstimulated HaCaT cells was relatively short (Figure 6), approximately 90–180 min, in general

Figure 7 Stability of PTHrP transcripts encoding each of the three alternative 3' exons in the presence of EGF

Total RNA from the same experiment depicted in Figure 6 was subjected to Northern-blot analysis and probed with cDNA probes specific for each of the three alternative 3' exons as described in the Experimental section. The upper panel demonstrates the induction of exon VII-containing transcripts in EGF-treated cells ($t = 0$ h) and its very slow decay after treatment with actinomycin D for the times indicated. The second panel demonstrates the basal expression of exon VIII-containing transcripts which are stable after actinomycin D treatment. There is a marked induction of exon VIII-containing transcripts in cells treated with EGF ($t = 0$ h) and again these are stable in the presence of actinomycin D. The basal expression of exon IX-containing transcripts was difficult to depict by autoradiography (third panel); however, we were able to quantify the signal in the PhosphorImager. We found that exon IX-containing transcripts had a half-life of 1.5 h in both control and EGF-treated cells where they were expressed to a slightly greater extent. The lower panel depicts the GAPDH signal corresponding to the upper panel for comparison.

agreement with previous reports [28,44]. However in experiments where EGF was first added to HaCaT cells for 2 h, followed by the addition of actinomycin D, PTHrP mRNA was considerably more stable, and the level was diminished by 50% only after 10–20 h of actinomycin D treatment (Figure 6). This effect appeared to be rather specific since the stability of *c-fos* mRNA, which is also up-regulated at the transcriptional level by EGF, was unchanged in the presence of EGF (Figure 6). The EGF-stimulated increase in the half-life of PTHrP mRNA was obtained in similar experiments where DRB was used as the transcriptional inhibitor (data not shown). To determine whether the ability of EGF to stabilize PTHrP mRNA was the same for transcripts containing each of the alternative 3' exons, Northern-blot analyses using cDNA probes specific for exons VII, VIII and IX were conducted. We found that the probes specific for exons VII and VIII produced strong hybridization signals in the EGF-treated samples and that these mRNA species were very stable. Only a very weak signal corresponding to transcripts containing exon VII was obtained in the control samples and it was not possible to determine the half-life of these species. However, strong signals corresponding to species containing exon VIII were present in control samples and these transcripts appeared to be constitutively stable (Figure 7). A probe complementary to exon IX produced only a weak signal in EGF-treated samples and this decayed rapidly with the same half-life as the signal in the control samples. Since the exonic probes were of different size and specific activity, we cannot deduce directly from the strength of the signals the relative abundance of the three populations of PTHrP mRNA. However, it can be inferred from these experiments that PTHrP mRNA species containing exons VII and VIII are stable in the presence of EGF while those containing exon IX are not. To study only post-transcriptional effects of

Figure 8 RT-PCR analysis of PTHrP promoter utilization

RNA was isolated from confluent HaCaT cells, treated for 3 h in the presence (E) or absence (C) of 1 nM EGF, as described in the Experimental section. (a) Ethidium bromide staining of a 2% (w/v) agarose gel showing the products obtained in each PCR. The sizes of the products are indicated along the left-hand side of the gel and were obtained by reference to DNA size markers (not shown). Primer combinations for each reaction are shown above the corresponding pairs of bands. P1, P2 and P3 indicates transcripts initiated by the P1, P2 and P3 promoters respectively, and V-VI indicates transcripts spanning the common exons V-VI. The smear below the 161 bp fragment in each lane represents unincorporated primers. (b) Southern hybridization of the gel depicted in (a). PCR products were transferred to a nylon filter and hybridized with [γ - 32 P]oligonucleotide obfr 15.8 to detect all PTHrP transcripts. Autoradiography was carried out using the same exposure time for all PCR products. The filter was subsequently stripped and re-probed with the [γ - 32 P]oligonucleotide, GAPDH-1, to compare the levels of GAPDH mRNA amplified from the same samples of RT-RNA in separate PCRs. This provided a measure of mRNA abundance and the efficiency of the reverse-strand synthesis. The amplification and detection of the 414 bp GAPDH fragments are shown on the right-hand side. This experiment was performed on preparations of RNA from three independent experiments with similar results. A semi-quantitative estimation of the effect of EGF on the activity of the three promoters was obtained by measuring the hybridization signals corresponding to each PCR product with a Molecular Dynamics PhosphorImager. Mean treated/control values were as follows: P1-initiated transcripts $T/C = 16.7 \pm 3.5$, P2-initiated transcripts $T/C = 2.9 \pm 0.8$, P3-initiated transcripts $T/C = 2.4 \pm 0.3$, and exon V to exon VI $T/C = 2.7 \pm 0.4$.

EGF, experiments were also conducted in which PTHrP mRNA levels were not first stimulated with EGF, instead actinomycin D (or DRB) was added at the same time as EGF. Again the half-life of PTHrP mRNA was markedly increased in cells exposed to EGF during the actinomycin D (or DRB) treatment (data not shown), suggesting that the stabilization of PTHrP mRNA was not dependent on prior EGF-stimulated transcription. Together with the results described earlier where cycloheximide failed to abrogate the EGF stimulation of PTHrP mRNA (Figure 3b), these data suggest that the actions of EGF were independent of the *de novo* synthesis of a signal-transducing molecule, mRNA-stabilizing protein or transcription factor. While such molecules are likely to mediate the EGF effects described here, we propose that their activation by EGF most likely involves post-translational modifications such as phosphorylation or intracellular translocation.

We also studied the effects of EGF on the abundance of PTHrP mRNA using RT-PCR. The sense strand-specific primers were designed to amplify transcripts specifically initiated by the P1, P2 and P3 promoters and the antisense strand-specific primer was designed to hybridize to a sequence in the common exon, exon VI (Figures 5a and 8a). While P1- and P3-initiated transcripts exclusively contain exons I and IV respectively [8,9], both P1 and P2 transcripts may contain exon III. To amplify transcripts specifically initiated by P2, we made use of the 11 bp region 5' to the splice acceptor site in exon III, which is unique to P2-initiated transcripts [14]. The sense strand-specific primer, obfr 15.95, contained the 16 most 5' nucleotides of P2-initiated transcripts and did not hybridize with P1-initiated transcripts

under the PCR conditions employed. The RT-PCR products were verified by Southern-blot analysis using a radiolabelled oligonucleotide probe (obrf 15.8) complementary to a sequence in exon VI, but not overlapping with the antisense strand-specific primer (Figures 5a and 8a). With this approach we identified products corresponding to utilization of all three promoters. The most dramatic effect of EGF was seen on products initiated by P1, where there was consistently about a 16-fold increase in the abundance of transcripts containing exon I (Figure 8). This contrasted markedly with the result we obtained in the transient transfection assays where EGF appeared to have little effect on the rate of P1 transcription. There was also about a 3-fold increase in the abundance of transcripts initiated by the GC-rich promoter, P2, and a 2.4-fold effect on transcripts initiated by P3. In summary, while we cannot interpret the RT-PCR data to comment on the relative usage of the three promoters in the PTHrP gene, the extent of EGF regulation was greatest on transcripts initiated by P1, then P2 and finally P3.

DISCUSSION

In the human keratinocyte cell line, HaCaT, elevated levels of PTHrP gene expression in response to EGF involved increases in both the rate of transcription and mRNA stability. Two direct approaches were used to demonstrate activation of transcription of the PTHrP gene: nuclear run-on assays and transient transfection assays. In nuclear run-on experiments, a 4-fold increase in the transcription rate of the PTHrP gene was observed in response to EGF. In transient transfection experiments, our results suggest that the increased transcription may be mediated largely by the GC-rich promoter, P2, with some contribution from P3, the downstream promoter. The RT-PCR experiments confirmed that levels of P2- and P3-initiated transcripts were stimulated by EGF, but in addition revealed striking effects of EGF on the abundance of P1-initiated transcripts. The marked increase in the number of transcripts containing exon I was independently confirmed by Northern hybridization with an exon I-specific cDNA probe (data not shown). These data were somewhat surprising in view of the fact that EGF did not regulate the activity of pSMR492, the CAT construct containing 355 bp of the P1 5' flanking sequence. One interpretation of this is that EGF increased the abundance of P1-initiated transcripts through an effect on mRNA stability only. However, it is also possible that our P1 construct did not extend far enough in the 5' direction (or in the 3' direction) to include all the regulatory elements normally conferring EGF responsiveness on this promoter. In a recent study by Campos et al. [49] RT-PCR was used to investigate relative promoter usage in samples of normal and neoplastic human tissue. These authors found that in most cases where the PTHrP gene was being expressed, both the upstream TATA promoter (P1) and the downstream TATA promoter (P3) were active (P2 was not analysed in this study), and there were no major quantitative differences in the pattern of promoter usage between normal and neoplastic tissues. However, our results indicate that, at least in response to EGF, the pattern of promoter usage may be markedly altered. Whether other growth factors and hormones regulating PTHrP gene expression evoke similarly complex responses remains to be determined.

EGF has been shown to stimulate the transcription of several other genes [50–53] and two signalling pathways linking the EGF receptor (EGF-R) to transcription factors have been proposed. Both pathways are initiated by autophosphorylation of the ligand-activated EGF-R. In the first, a cascade of phosphorylation events links the protein tyrosine kinase receptor to serine/threonine kinases, producing activation of transcription factors

in the nucleus and their interaction with specific response elements in the DNA (for review see [54]). In the second pathway, cytoplasmic transcription factors are directly phosphorylated by the EGF-R itself. In this 'direct effector' model, the activated factors are then translocated to the nucleus where they form active transcriptional complexes on target genes [55]. For example, the transcription factor p91 is directly phosphorylated by the EGF-R in a ligand-dependent manner and produces activation of the *c-fos* promoter upon binding to the SIE (*c-sis*-inducible element) [56]. At present we cannot conclude that one or both of these mechanisms mediate the transcriptional effects of EGF reported here. However, our finding that the action of EGF on PTHrP gene expression was not inhibited by cycloheximide in HaCaT cells is compatible with both of the mechanisms described above, since neither requires *de novo* protein synthesis.

EGF was also found to have a marked effect on PTHrP mRNA stability, again through mechanisms independent of *de novo* mRNA and protein synthesis. EGF-mediated increases in mRNA stability have been reported previously [57–59], most notably in the case of EGF-R mRNA [57]. All transcripts of the PTHrP gene contain AUUUA-rich motifs in their 3' untranslated regions (UTRs) which are thought to confer instability upon mRNA (for review see [60]), contributing to the notion that PTHrP is a member of the 'immediate early' gene family [28]. Surprisingly, our analysis with exon-specific probes revealed that 3' alternative splicing produced species of human PTHrP mRNA that differed in stability. We found that exon VIII-containing PTHrP transcripts were stable in HaCaT cells treated with actinomycin D, whether EGF was present or not. We could not detect exon VII-containing transcripts in control cells but the markedly elevated levels of exon VII-containing transcripts produced in response to EGF were stable in the presence of actinomycin D. In contrast, exon IX-containing transcripts had a relatively short half-life (Figure 7) and though weakly stimulated by EGF, they continued to decay at about the same rate as those in unstimulated cells. Inspection of the nucleotide sequence in the three alternative 3' UTRs revealed the presence of four AUUUA motifs in both exons VII and IX, with three out of the four being closely clustered in both cases. Meanwhile only two more widely spaced AUUUA motifs are present in exon VIII. It has been proposed that the ability of the AU-rich sequence binding proteins, AU-BPs, to form complexes with AUUUA motifs and target these RNAs for rapid degradation may depend on at least three motifs being in close proximity to one another [61,62]. This might explain why exon VIII-containing transcripts are apparently intrinsically more stable than those containing exon IX (and possibly exon VII). All these data suggest that the ability of EGF to increase PTHrP mRNA stability may depend on at least two mechanisms, one involving the post-translational (or co-translational) stabilization of PTHrP mRNA and the second arising from altered patterns of 3' alternative splicing. For example, transcripts produced by the EGF-activation of PTHrP promoters may be more likely to retain the 'stable' exon VIII than those produced constitutively.

The phenomenon of differential PTHrP mRNA stability may be unique to human PTHrP. While the expression of the rat PTHrP gene is also up-regulated by EGF [28,63], the 3-fold increase in PTHrP mRNA levels is less than the 10-fold increase observed in the present study. Interestingly, the rat gene does not contain 3' exons corresponding to exons VII and VIII in the human [9], which we have demonstrated here to be stable in the presence of EGF. Thus, the more marked responsiveness of the human PTHrP gene may be due partly to the effect of EGF on PTHrP mRNA stability.

We have shown that EGF regulation of human PTHrP gene expression is a complex, multifactorial process involving increases in both gene transcription and the stability of PTHrP mRNA. The interaction of these two mechanisms produces dramatically elevated expression of PTHrP which, at least in HaCaT cells, favours the accumulation of transcripts containing exon I at the 5' terminus and either exon VII or exon VIII at the 3' terminus. This would predict the elevated production of the 139 and 173 amino acid isoforms of PTHrP, relative to the 141 amino acid isoform encoded by exon IX-containing transcripts. The significance of this is largely contingent on whether the three isoforms of PTHrP differ in their biological properties. However, this has not been established so far, and indeed we do not know whether intact, full-length PTHrP molecules are secreted or subjected to intracellular processing. Our results suggest that the evolution of a human PTHrP gene containing three alternative 3' exons may have advanced not only to generate the specific isoforms of the protein, but also to permit up-regulation of PTHrP expression through the production of stable forms of mRNA. It remains to be determined whether the regulatory mechanisms invoked here by EGF also underlie the overproduction of PTHrP in squamous carcinoma and other tumours in which humoral hypercalcaemia is a frequent complication of malignancy.

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