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Nucleolar Localization of Parathyroid Hormone-Related Peptide Enhances Survival of Chondrocytes under Conditions That Promote Apoptotic Cell Death

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Parathyroid hormone-related peptide (PTHrP) is a mediator of cellular growth and differentiation as well as a cause of malignancy-induced hypercalcemia. Most of the actions of PTHrP have been attributed to its interaction with a specific cell surface receptor that binds the N-terminal domain of the protein. Here we present evidence that PTHrP promotes some of its cellular effects by translocating to the nucleolus. Localization of transiently expressed PTHrP to the nucleolus was dependent on the presence of a highly basic region at the carboxyl terminus of the molecule that bears homology to nucleolar targeting sequences identified within human retroviral (human immunodeficiency virus type 1 and human T-cell leukemia virus type 1) regulatory proteins. Endogenous PTHrP also localized to the nucleolus in osseous cells in vitro and in vivo. Moreover, expression of PTHrP in chondrocytic cells (CFK2) delayed apoptosis induced by serum deprivation, and this effect depended on the presence of an intact nucleolar targeting signal. The present findings demonstrate a unique intracellular mode of PTHrP action and a novel mechanism by which this peptide growth factor may modulate programmed cell death.

Peptide growth factors have been shown to associate with nuclei (15, 26, 52, 53) and at times nucleoli (8, 13, 39, 46) of target cells. This direct association may represent an alternative mechanism to the classical signal transduction pathway whereby growth-related proteins can modulate cellular function. How these peptides reach the nucleus remains unclear. In most cases, nuclear localization has been demonstrated following internalization of ligand-receptor complexes, suggesting that receptor-mediated endocytosis may allow the ligand access to the cytoplasmic compartment for subsequent transport to the nucleus. Alternative initiation of translation (2) and alternative splicing (39) have also been invoked as mechanisms to generate protein forms which would preferentially translocate to the nucleus rather than be directed to the secretory pathway.

Regardless of size, proteins functioning within the nucleus possess a nuclear localization signal (NLS) which is required for recognition by and active translocation through the nuclear pore (for reviews, see references 51 and 59). Subsequent subnuclear compartmentalization appears to require additional structural information. Sequences involved in nucleolar targeting have been examined in a number of eukaryotic proteins such as nucleolin (55), N038 (49), the transcription factor UBF (38), and HSP70 (44), as well as in three human retroviral regulatory proteins, human immunodeficiency virus type 1 (HIV-1) Tat (17), HIV-1 Rev (16), and human T-cell leukemia virus type 1 (HTLV-1) Rex (60). A consensus nucleolar targeting signal (NTS), consisting of an NLS associated with longer arrays of basic amino acids, has been proposed for the viral proteins (17). Effective localization of most eukaryotic proteins to the nucleolus, however, appears to be dependent on the presence of an NLS, together with additional functional domains (55, 68).

Little is presently known about the events that determine the timing and degree of nucleolar translocation or the role that it may serve in normal cellular function. In some instances, it appears to be related to progression through the cell cycle (8, 13, 46) and, in the case of basic fibroblast growth factor (bFGF), to activation of ribosomal gene transcription (13).

Parathyroid hormone-related peptide (PTHrP) is a tumor-derived, secretory protein which is structurally related to parathyroid hormone (PTH), the major regulator of calcium homeostasis (for a review, see reference 43). PTH and PTHrP bind to a common G-protein-coupled cell surface receptor that recognizes the N-terminal (positions 1 to 34) region of the molecules (1, 29). Hence, when tumor-derived PTHrP enters the circulation, it activates receptors in bone and kidney, thereby eliciting PTH-like bioactivity and giving rise to the common paraneoplastic syndrome of malignancy-associated hypercalcemia (for a review, see reference 14).

The human gene for PTHrP has been mapped to the short arm of chromosome 12 and spans more than 15 kb of genomic DNA (40, 69). By alternate splicing, it can serve as template for three isoforms of preproPTHrP, giving rise to mature forms composed of 139, 141, or 173 amino acids. In rodents, only one major form of PTHrP is produced; it is composed of 141 amino acids in the rat (33, 63) and 139 amino acids in the mouse (40).

Unlike PTH, PTHrP does not circulate in appreciable amounts in normal subjects but is widely expressed in a number of fetal and adult tissues, where it is thought to act in a paracrine/autocrine manner (for reviews, see references 14 and 22). Both in vitro (24, 30, 31) and in vivo (67), PTHrP has been shown to influence keratinocyte growth and differentiation.

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Overexpression of PTHrP in the skin of transgenic mice results in epidermal hyperkeratosis and abnormal hair follicle differentiation (67). On the other hand, mice homozygous for PTHrP gene ablation exhibit skeletal deformities that are due, in part, to diminished proliferation and inappropriate differentiation of chondrocytes in the developing skeleton (4, 32).

Although structural similarities between PTH and PTHrP account for their binding to the same cell surface receptor, it is perplexing that a single receptor should mediate both the endocrine regulation of calcium homeostasis by PTH and the local paracrine/autocrine activities of PTHrP. In this study, we therefore examined the possibility that some of the actions of PTHrP involve alternate pathways. We report here that PTHrP contains an NTS that bears homology to sequences in human retroviruses (HTLV-1 and HIV-1) shown to target viral regulatory proteins to the nucleolus. The PTHrP NTS is functional and capable of translocating a heterologous cytoplasmic protein, β-galactosidase, to the nucleolus when expressed as a fusion protein in COS-7 cells. Moreover, we present evidence that in vitro as well as in vivo, endogenous PTHrP localizes to the nucleolus of murine bone cells. Finally, we demonstrate that expression of PTHrP forms containing the NTS prolongs survival of chondrocytic cells under conditions that promote programmed cell death. These findings are consistent with the hypothesis that in addition to activation of classical signal transduction pathways, PTHrP accomplishes some of its biological effects following translocation to the nucleolus.

MATERIALS AND METHODS

Construction of plasmids. The 1.2-kb rat PTHrP (rPTHrP) cDNA (clone rPLPm 10 [70]) was inserted in the *Eco*RI site of the polylinker of the mammalian expression vector pcDNA1 (Invitrogen, San Diego, Calif.), generating plasmid pPTHrP. The orientation was verified by restriction enzyme digestion and DNA sequencing. The 3′ untranslated region of pPTHrP was removed by digestion with *Bgl*II, treatment with Klenow enzyme, and then cutting at the unique *Eco*RV site in the polylinker to release the 3′ untranslated region. The blunt ends were subsequently religated to form plasmid pPTHrP/1. A 0.7-kb fragment of PTHrP cDNA, lacking the 3′ untranslated region, was generated by cutting pPTHrP with *Bgl*II, treating it with Klenow enzyme, and then cutting with *Hin*dIII. This fragment was then inserted into the mammalian expression vector pcDNA3 (Invitrogen), generating plasmid pPTHrP/3 for use in stable transfections.

Plasmid pΔ-36+1/1 (PTHrP without the prepro sequence) was created by using oligonucleotides N1 (5′GCC ATG GCG GTG TCT GAG CAC CAG CT 3′) and N2 (5′ GTT <u>TCA</u> ATG CGT CCT TGA GCT GGG CT 3′). N1 contains an ATG codon in the optimal context for initiation of translation (35) followed by sequences encoding the first six amino acids of the mature rPTHrP protein; N2 contains the last seven codons, including the termination codon (underlined), of rPTHrP. Using PCR, oligonucleotides N1 and N2 were used to amplify sequences from plasmid pPTHrP/1. The amplified product consisted of a methionine codon followed by sequences encoding the full-length mature rPTHrP protein. This fragment was then ligated into the unique *Eco*RV site of pcDNAI. For stable transfections, the 0.4-kb *Hin*dIII-*Xba*I PTHrP fragment from pΔ-36+1/1 was inserted into pcDNA3, generating pΔ−36+1/3.

Plasmid pΔ-36+1Δ87-107/1 was generated by cutting pΔ-36+1/1 with *Sma*I and *Tth*111I, treating it with Klenow enzyme, and religating the blunt ends. This procedure removed the putative NTS from the mature PTHrP protein. Plasmid pΔ87-107/1 was similarly derived from pPTHrP/1. For stable transfections, the *Eco*RI-*Xho*I PTHrP fragment from pΔ87-107/1 was inserted into pcDNA3, generating pΔ87-107/3.

Plasmid pβgal was constructed by ligating the *Not*I fragment of plasmid pCMVβ (Promega, Madison, Wis.), containing the *Escherichia coli lacZ* gene, into the unique *Not*I site of the pcDNAI polylinker. Orientation was verified by restriction digest.

Plasmid p87-107βgal was generated by PCR amplification of pΔ-36+1/1 sequences, using N3 (5′ GCC ATG GCG <u>CCC GGG</u> GTG TCT GAG CAC CAG CTA CTG CA 3′), containing an initiation codon followed by an Ala codon and a *Sma*I restriction site (underlined), as the 5′ oligonucleotide and N2 as the 3′ oligonucleotide. The amplified fragment was inserted into the unique *Eco*RV site in the polylinker of pcDNA1, and the resulting plasmid was cut with *Sma*I and religated, bringing the putative NTS in frame with the +2 Ala codon. The construct was then restricted with *Tth*111I, blunt ended with mung bean nuclease, and cut with *Xho*I prior to ligation with a *Sma*I-*Sal*I fragment derived from plasmid pMC-1871 (Pharmacia LKB, Uppsala, Sweden), containing the *E. coli lacZ* gene missing the first eight nonessential codons. The in-frame fusion protein was verified by DNA sequencing.

Plasmid pΔ-36+1Δ87-141βgal was derived by ligating the *Sma*I-*Sal*I fragment from pMC-1871 into pΔ-36+1, which was cut with *Sma*I and *Xho*I. Plasmid pΔ-36+1Δ108-141βgal was constructed by ligating a *Hin*dIII-*Sma*I fragment from construct pΔ-36+1/1 to plasmid p87-107βgal which had been restricted with *Hin*dIII and *Sma*I.

COS-7 cell culture and transient transfection. COS-7 cells were maintained in Dulbecco's modified Eagle medium (DMEM; Life Technologies Inc., Grand Island, N.Y.) supplemented with L-glutamine, 10% heat-inactivated calf serum, and antibiotic-antimycotic (Life Technologies). Twenty-four hours prior to transfection, cells were plated at a density of 3×10^5 per well in six-well cluster plates. Cells were washed twice with phosphate-buffered saline (PBS) and incubated with 1 μg of plasmid DNA in 1 ml of DMEM containing 0.1% bovine serum albumin (BSA; Sigma, St. Louis, Mo.), 400 μg of DEAE-dextran (Pharmacia LKB), and 0.1 mM chloroquine (Sigma) for 3.5 h at 37°C. Transfected cells were shocked with 10% dimethyl sulfoxide (Sigma) in PBS for 2 min and cultured in supplemented DMEM for 48 h prior to replating (30,000 cells per cm^2) on glass coverslips coated with 0.1% gelatin. Indirect immunofluorescence was performed 24 h later (72 h posttransfection).

Indirect immunofluorescence. Cells were washed twice with PBS, fixed and made permeable for 3 min in ice-cold methanol-acetone (1:1), rehydrated for 15 min in PBS, and blocked with 2% normal goat serum (Sigma) in PBS for 30 min. After being washed with PBS containing 0.2% Triton X-100 (PBST), cells were incubated for 1 h at room temperature with one of the primary antisera diluted in PBST containing 2% normal goat serum, washed several times with PBST, and incubated for 1 h with the appropriate fluorescein isothiocyanate (FITC)- or tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibody diluted in PBST containing 2% normal goat serum. Coverslips were then washed extensively with PBST and mounted in 75% glycerol containing 2.5% 1,4-diazabicyclo-[2.2.2]octane (DABCO; Sigma) and examined with a Leitz Aristoplan microscope (Leica Heerbrugg, Heerbrugg, Switzerland).

Antibodies. The polyclonal antisera to rPTHrP(1-34) (rPTHrP containing amino acids 1 to 34), rPTHrP(67-86), and rPTHrP(87-110) were raised in a rabbit, using synthetic peptides conjugated to metBSA. A monoclonal antibody to recombinant human PTHrP(1-141) which recognizes the 34-68 epitope was purchased from Oncogene Science Inc., Uniondale, N.Y. Anti-nucleolar antigen serum from a patient with progressive systemic sclerosis was a kind gift from Peter Small. FITC-conjugated anti-mouse immunoglobulin G (IgG; Fc specific), FITC-conjugated anti-rabbit IgG, and TRITC-conjugated anti-human IgG/M/A antisera, all generated in goats, as well as mouse anti-β-galactosidase fractionated ascites fluid, were purchased from Sigma Immunochemicals.

In vitro translation. cRNAs were transcribed from *Xho*I-linearized plasmids by using T7 RNA polymerase. In vitro translation was performed by using a rabbit reticulocyte lysate cell-free system as previously described (7). Translation products labeled with [^{3}H]leucine were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), using a 17% polyacrylamide gel, and autoradiography.

Metabolic labeling and immunoprecipitation of proteins in COS-7 cells. COS-7 cells were plated at a density of 2×10^6 in 100-mm-diameter dishes 24 h prior to transfection with 5 μg of plasmid DNA with DEAE-dextran. Cells were split into two 100-mm dishes at 48 h postransfection and labeled with [^{3}H]leucine at 72 h posttransfection. Dishes were washed twice with leucine-free medium (Life Technologies) and incubated for 6 h in leucine-free medium containing 175 μCi of [^{3}H]leucine (specific activity, 157 Ci/mmol; ICN Biomedicals, Mississauga, Ontario, Canada), 0.1% BSA, and 2.5 mM phenylmethylsulfonyl fluoride. Dishes were rinsed twice with cold PBS and placed on ice, and the cell layers were scraped into 500 μl of radioimmunoprecipitation assay buffer containing 10 mM Tris (pH 7.2), 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 5 mM EDTA, 0.02% sodium azide, 150 mM NaCl, and phenylmethylsulfonyl fluoride (final concentration, 500 μg/ml). Lysates were passed several times through a 21-gauge needle and centrifuged for 30 min at 4°C, and the supernatant was stored at −80°C. Samples were precleared by using IgGsorb (The Enzyme Center, Malden, Mass.) prepared as instructed by the manufacturer. Immunoprecipitation of precleared supernatants was performed with an antiserum raised in rabbit against rPTHrP(1-34) at a dilution of 1:250. Precipitated proteins, approximately 30,000 cpm per lane, were then loaded onto a 17% polyacrylamide gel. Fluorography was performed with En^{3}Hance (NEN Research Products, Boston, Mass.), and the gels were dried and exposed to XAR-5 film (Eastman Kodak Company, Rochester, N.Y.) for 24 to 48 h. Molecular weight markers were run in parallel, stained with Silver Stain Plus (Bio-Rad, Hercules, Calif.), and dried along with the labeled gels.

Extraction and immunostaining of osteoblast-like cells. Calvariae from 2-day-old CD1 mice (Charles River) were dissected free of fibrous and cartilagenous tissue and cut into small fragments prior to collagenase (Worthington Biochemical Co., Freehold, N.J.) digestion as previously described (62). Cells released by the digestion were cultured in DMEM supplemented with 10% heat-inactivated calf serum until approximately 50% confluent, at which time they were fixed and stained immunochemically for PTHrP as described previously (4).

Preparation, embedding, and immunogold labeling of murine tibiae. Tibiae extracted from 18.5-day-old fetal mice, either wild type or homozygous for PTHrP gene ablation through targeted disruption by homologous recombination

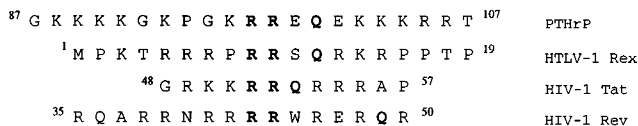

FIG. 1. Sequence homology of putative NTSs. The amino acid sequence of the putative NTS in PTHrP was aligned with those described for a number of human retroviral regulatory proteins. Shared homology includes an arginine hinge with an adjacent glutamine (boldface) flanked by basic amino acids. Numbers indicate the positions of amino acid residues within the respective mature proteins.

(32), were fixed and decalcified as described previously (66) prior to embedding in glycolmethacrylate. Sections of glycolmethacrylate-embedded material were either stained immunochemically for light microscopic examination or labeled with immunogold (Sigma) for electron microscopic examination. Immunoreactive elements were detected with a JEOL 2000FX electron microscope operated at 80 kV.

CFK2 cell culture and stable transfection. CFK2 chondrocytes were maintained in culture in RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotic-antimycotic. Twenty-four hours prior to transfection, cells were replated at a density of 10^6/10 ml of supplemented medium in 100-mm-diameter dishes. Transfection mix (1 ml) containing 10 μg of plasmid DNA, 250 mM $CaCl_2$, 0.75 mM Na_2HPO_4, 5 mM KCl, 140 mM NaCl, 6 mM glucose, and 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.12) was added to each dish, and the dishes were incubated for 16 h. After washing with PBS, fresh supplemented RPMI 1640 was added and incubations continued for 48 h, at which time the cells were replated in 150-mm-diameter dishes in medium containing 700 μg of G418 (Life Technologies) per ml. Selection was continued for 21 days, with medium being changed every 3 to 4 days. Resistant colonies were trypsinized, and populations were expanded in medium containing 350 μg of G418 per ml prior to use.

DNA fragmentation assay. CFK2 chondrocytes stably transfected with pcDNA3 vector were plated at a density of 10^6/100-mm-diameter dish in RPMI 1640 supplemented with 10% FBS. Culture medium was replaced with RPMI 1640 supplemented with 2% FBS after 16 h. Twenty-four hours later, cell layers were washed twice with serum-free RPMI 1640 and then cultured in serum-free RPMI 1640 for the indicated time period. After 24 h of serum-free culture, nonadherent material was removed from medium conditioned by the CFK2 population and processed for assessment of DNA fragmentation (see below). Genomic DNA was harvested from adherent cells by scraping the cells into RPMI 1640, washing them with PBS, and incubating them for 2 h at 55°C in 50 μl of buffer containing 10 mM Tris (pH 8), 2 mM EDTA, 400 mM NaCl, 0.5% SDS, and 50 ng of proteinase K (Boehringer Mannheim, Mannheim, Germany) per μl. Samples were extracted once with phenol, precipitated with ethanol, reconstituted in 20 μl of sterile water containing 2 μg of RNase A (Boehringer Mannheim), and incubated for 15 min at 37°C. Genomic DNA (15 μg) was loaded into each lane of a 1% agarose gel stained with ethidium bromide.

Assessment of apoptotic death. CFK2 chondrocytes stably transfected with vector or with the various PTHrP constructs were photographed 24 h after plating in medium containing 10% FBS and after 9 days of serum-free culture, using a Leitz Diavert phase-contrast microscope equipped with a Wild MPS Photoautomat (Leica). Quantitative assessment of cell death was made by counting cells, in the presence of trypan blue, at timed intervals following serum withdrawal. Control cultures (transfected with vector alone) were maintained both in the absence and in the presence of 10^{-9} M PTHrP(1-34) for these experiments. CFK2 cells stably transfected with pcDNA3 vector were prepared for electron microscopic examination using standard procedures. Epon-embedded thin sections were visualized with a JEOL 2000FX electron microscope at 80 kV.

RESULTS

Characterization of the NTS in PTHrP. Examination of the amino acid sequence of the mature PTHrP protein revealed that residues 87 to 107 have homology with NTSs in human retroviral regulatory proteins HIV-1 Tat, HIV-1 Rev, and HTLV-1 Rex. In all four proteins, an arginine hinge, with an adjacent glutamine residue, is flanked by stretches of basic amino acids (Fig. 1).

To determine whether the putative NTS within PTHrP is functional, an expression plasmid containing the rPTHrP cDNA was constructed and transfected into COS-7 cells. The subcellular localization of the transiently expressed protein was then examined by indirect immunofluorescence using a monoclonal antibody that recognizes the 34-68 epitope of human PTHrP (Oncogene Science). As anticipated for a secretory protein, the distribution of PTHrP in the majority of transfected cells was in a reticular pattern indicative of the secretory pathway (Fig. 2a). However, in about 15% of transfected cells, both nucleolar and reticular cytoplasmic staining was observed (Fig. 2b), suggesting that the putative NTS may indeed be functional. These results obtained with the monoclonal antibody were verified by using two different polyclonal antisera, raised against synthetic human PTHrP (1-34) and hPTHrP(67-86). The identical staining pattern was seen with all three antibodies confirming the specificity for PTHrP.

Native PTHrP contains a prepro sequence (leader sequence) which targets the nascent protein to the endoplasmic reticulum for secretion. Since translocation-competent binding occurs cotranslationally, the presence of the leader sequence would preferentially target the protein to the secretory pathway. We therefore constructed plasmids expressing leaderless forms of PTHrP to study the ability of the putative NTS to direct transport to the nucleolus. This was accomplished by introducing an ATG codon in the optimal context for eukaryotic initiation of translation followed by sequences encoding the mature PTHrP protein. The resulting proteins would therefore resemble mature PTHrP following cleavage of the prepro peptide. In striking contrast to the reticular cytoplasmic localization of the full-length protein, the leaderless form localized almost exclusively to the nucleus in what appeared to be a nucleolar pattern in more than 90% of transfected cells (Fig. 2c). Deletion of sequences encoding the putative NTS from the construct expressing the leaderless protein resulted in purely cytoplasmic staining (Fig. 2d). A similar deletion of the putative NTS from the construct expressing full-length PTHrP elicited an exclusively reticular cytoplasmic localization of the expressed protein (Fig. 2e). These results suggested that in the absence of the prepro peptide, PTHrP could be preferentially directed to the nuclear compartment and that the absence of the NTS effectively abolished intranuclear localization of the recombinant protein.

To verify that the intranuclear organelle to which PTHrP localized was the nucleolus, COS-7 cells were again transfected with the plasmid expressing the leaderless form of PTHrP. Cultured cells were then fixed and reacted simultaneously with rabbit antiserum directed against PTHrP(1-34) and with serum containing antibodies to nucleolar antigen obtained from a patient with progressive systemic sclerosis (21). Visualization of the nucleolar antigen by using TRITC-conjugated anti-human IgG/M/A revealed a nucleolar pattern of staining evident in all COS-7 cells (Fig. 3A). Identification of recombinant PTHrP protein by using FITC-conjugated anti-rabbit IgG demonstrated the same nucleolar pattern of staining only in transfected cells (Fig. 3B). These experiments verified the colocalization of the nucleolar antigen and transiently expressed mature PTHrP in COS-7 cells.

The ability of the putative PTHrP NTS to target a heterologous cytoplasmic protein to the nucleolus was tested by using a series of plasmids containing N-terminal PTHrP sequences fused in frame with the *E. coli lacZ* gene. When expressed in mammalian cells, β-galactosidase normally remains in the cytoplasm (Fig. 4a). When fused to the N-terminal 87 amino acids of the mature PTHrP form, β-galactosidase expression was again localized to the cytoplasm (Fig. 4b). In contrast, when fused to the N-terminal 107 amino acids, which include the putative NTS, the staining pattern was both nucleolar and cytoplasmic (Fig. 4c). The ability of the putative NTS to independently target a heterologous, cytoplasmic protein to the nucleolus was demonstrated by fusing amino acids 87 to 107 of

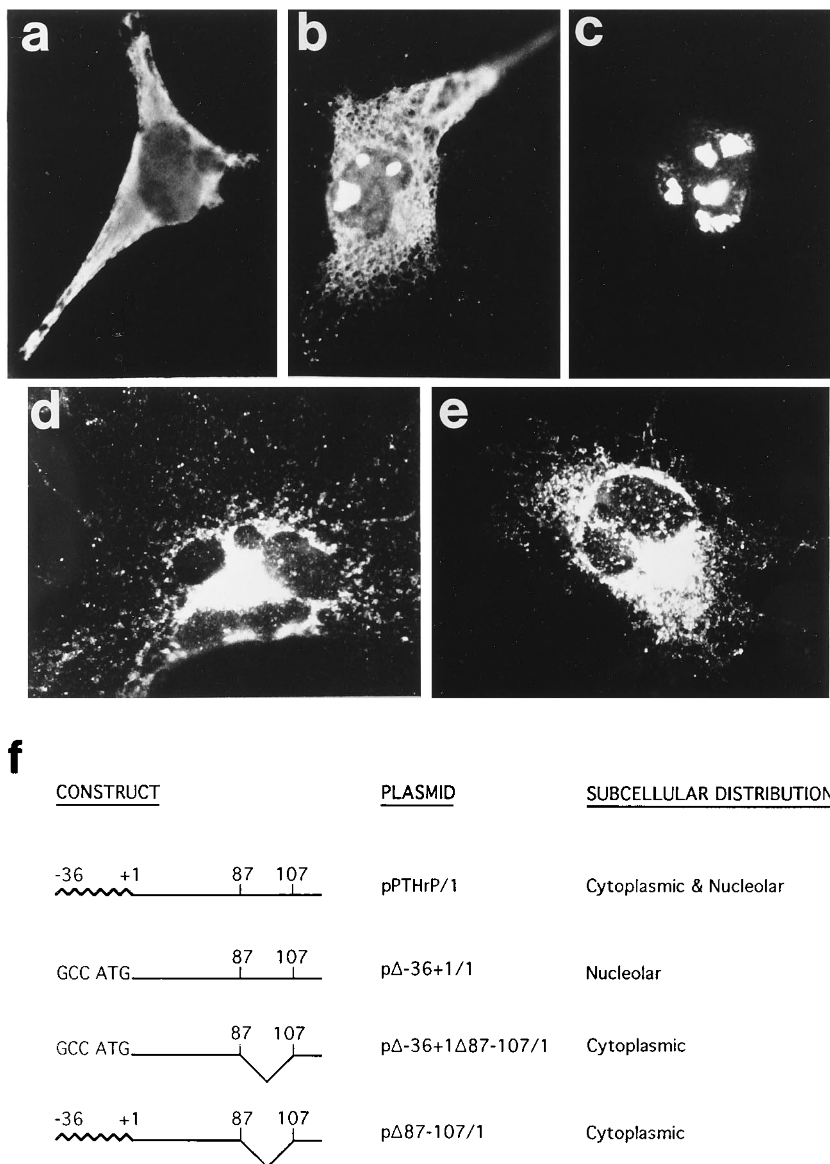

FIG. 2. Subcellular distribution of PTHrP in transfected COS-7 cells. Expression plasmids containing PTHrP having either an intact coding region or deletions within the coding region were transfected into COS-7 cells. (a to e) Subcellular distribution of transiently expressed proteins which were localized by indirect immunofluorescence, using a monoclonal antibody that recognizes amino acids 34 to 68 of human PTHrP. Plasmid transfections were as follows: (a and b) pPTHrP/1, (c) pΔ-36+1/1, (d) pΔ-36+1Δ87-107/1, and (e) pΔ87-107/1. (f) Plasmid constructs and subcellular distribution of the expressed proteins. Solid lines represent PTHrP sequences. Amino acids −36 to +1 encompass the prepro sequence, and amino acids 87 to 107 encompass the putative NTS of PTHrP. In plasmid names, Δ designates deleted sequence and /1 indicates the use of pcDNA1 as the vector. Magnification, ×400.

PTHrP to β-galactosidase. Virtually all cells transfected with this construct showed distinct nucleolar localization of the recombinant chimeric protein along with some cytoplasmic staining (Fig. 4d), thus confirming the functionality of the NTS.

Immunoprecipitation analysis of PTHrP from transfected COS-7 cells. The appropriate expression of wild-type and mutant PTHrP proteins in COS-7 cells was verified by using lysates from transfected cells labeled with [^{3}H]leucine. Proteins were immunoprecipitated with rabbit antiserum raised against PTHrP(1-34) and analyzed by SDS-PAGE. Figure 5A shows the radiolabeled products from in vitro translation of cRNAs transcribed from the linearized plasmids that were used to verify the nature of the immunoprecipitated proteins. As shown in Fig. 5B, transfection with plasmid pPTHrP/1 generated two main products, p25 and p21.5, corresponding to full-length PTHrP and the processed form following cleavage of the signal peptide, respectively. Mature PTHrP from both the COS-7 cell lysate (Fig. 5B) and the reticulocyte lysate cell-free system (Fig. 5A) migrated anomalously, with an M_r higher than that predicted from the cDNA (21,500 versus 16,100). This aberrant migration has been noted previously with recombinant PTHrP(1-141) (64) as well as with other nucleolar proteins (reference 61 and references therein) and has been attributed to the high content of basic amino acids. Transfection with plasmid pΔ87-107/1, expressing full-length PTHrP missing the NTS sequence, also generated products corresponding to full-length and processed forms of the nascent protein. In contrast, COS-7 cells transfected with plasmids expressing the

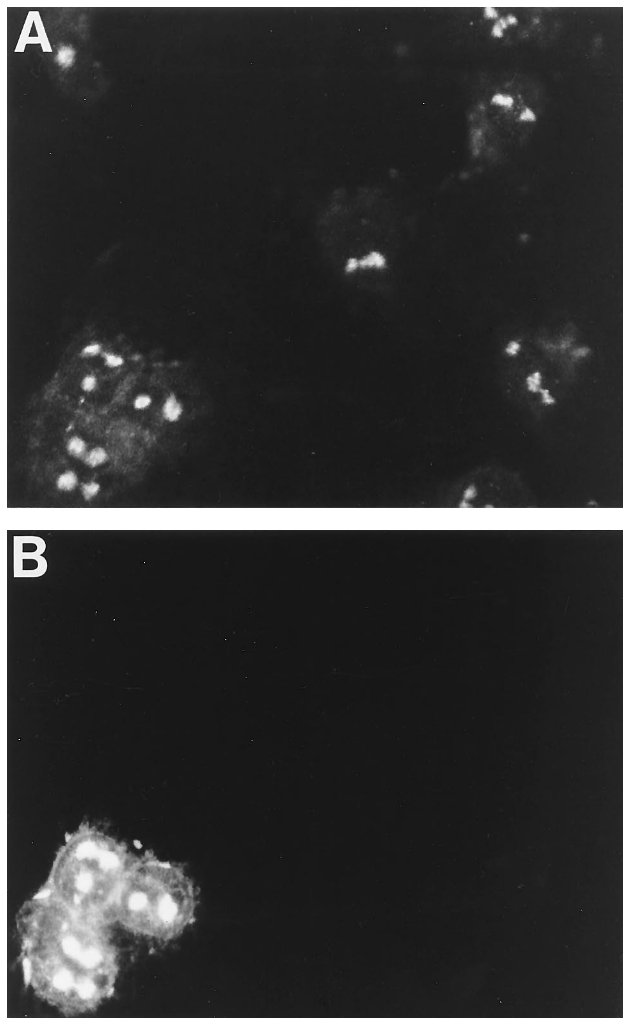

FIG. 3. Colocalization of anti-nucleolar antigen and PTHrP in COS-7 cells. Cells transfected with plasmid pΔ−36+1/1 were reacted simultaneously with anti-nucleolar antigen serum obtained from a patient with progressive systemic sclerosis and with rabbit antiserum raised against PTHrP(1-34). Immunoreactive complexes were detected with TRITC-conjugated anti-human IgG/M/A secondary antibody (identifying endogenous nucleolar antigen) (A) and FITC-conjugated anti-rabbit IgG secondary antibody (identifying transiently expressed PTHrP) (B). Magnification, ×400.

leaderless forms of PTHrP (pΔ-36+1/1 and pΔ-36+1Δ87-107/1) generated a single product. These results indicate that transient expression of recombinant forms of PTHrP in COS-7 cells results in generation of the expected protein products.

Endogenous PTHrP localizes to the nucleolus. The observations in studies using COS-7 cells clearly demonstrated that the NTS in the transiently expressed protein was indeed functional. PTHrP is expressed endogenously in a number of normal adult and fetal tissues, including murine bones. In the fetal skeleton, PTHrP is expressed in growth plate chondrocytes and osteoblasts (4). We therefore chose to study the subcellular localization of PTHrP in cultured osteoblast-like cells derived from calvariae of 2-day-old mice. Using a panel of antisera raised in rabbit against synthetic PTHrP fragments, we detected immunoreactivity in the cytoplasm of ~90% of cells and in both cytoplasmic and nuclear compartments of ~10% of cells. Cytoplasmic staining appeared diffuse, with a strong perinuclear halo in numerous cells, whereas the punctate nuclear staining was more intense and concentrated in nucleoli (Fig. 6A).

To further characterize the nucleolar localization of endogenous PTHrP, we examined plastic-embedded sections prepared from murine fetal long bones. Thick sections were stained immunochemically for light microscopic examination to localize regions of PTHrP-positive cells prior to cutting of thin sections for electron microscopic examination. Figure 6B shows immunogold labeling of an osteoblast nucleolus showing gold particles localized to the coarse fibrillar component, which is thought to represent newly transcribed rRNA. Although gold particles were occasionally found elsewhere in the nucleus, they never appeared in large numbers or associated with distinct structures. The specificity of this labeling for PTHrP was verified by examination of sections from long bones of fetal mice homozygous for ablation of the PTHrP gene following targeting by homologous recombination (32). No detectable PTHrP reactivity was observed in the cytoplasm or nucleoli (Fig. 6C) of cells in these sections.

Presence of the PTHrP NTS improves survival of CFK2 chondrocytes undergoing programmed cell death. During endochondral bone formation, chondrocytes follow an orderly progression through differentiation and are thought to die by apoptosis as capillaries and bone-forming cells invade the region (20). As both PTHrP and its N-terminal receptor have been localized in a spatially restricted manner in the growth cartilage of normal mice (5, 36), it is not surprising that chondrocytes in mice homozygous for PTHrP gene ablation demonstrated abnormalities in proliferation and differentiation. These observations are consistent with a role for PTHrP in modulating the process of endochondral bone formation and perhaps the apoptotic death of chondrocytes. We therefore examined the possibility that nucleolar localization of PTHrP influences cell survival, using the previously characterized chondrocytic cell line CFK2 (10, 11). Neither PTHrP mRNA (23) nor PTHrP protein (see Fig. 8A) has been detected in these cells in either the undifferentiated or differentiated state under normal circumstances. Following stable transfection with pcDNA3 vector (see below), apoptosis was induced in these cells by withdrawal of serum from the culture medium (18, 27). Programmed cell death commenced within hours in scattered cells, with loss of cell-cell contact followed by cytoplasmic and nuclear condensation. After 6 days of serum deprivation, light microscopic examination of cultures revealed few remaining adherent cells and numerous refractile bodies (Fig. 7A). DNA harvested at 24 h from nonadherent cells demonstrated the fragmentation characteristic of apoptosis. Figure 7B shows the orderly laddering of chromosomal DNA, generated by internucleosomal cleavage, into bands corresponding to multiples of approximately 180 bp. In contrast, DNA harvested from the adherent cell layer maintained its integrity and migrated as a single, high-molecular-weight band. The morphological characteristics of apoptosis (for a review, see reference 56) were confirmed by electron microscopic examination of cultures deprived of serum for 6 days. Numerous cells possessing few organelles, condensed chromatin, minimal cytoplasm, and membrane blebbing were observed (Fig. 7C).

To determine whether the PTHrP NTS is involved in programmed cell death induced by serum withdrawal, we established populations of CFK2 cells stably transfected with full-length preproPTHrP (pPTHrP/3), the nonsecretory mature form of PTHrP (pΔ-36+1/3), or preproPTHrP missing the NTS (pΔ87-107/3). Cells transfected with vector alone (pcDNA3) were used as controls. Immunochemical localization of PTHrP in these cell populations is shown in Fig. 8. Staining patterns were similar to those seen in transiently

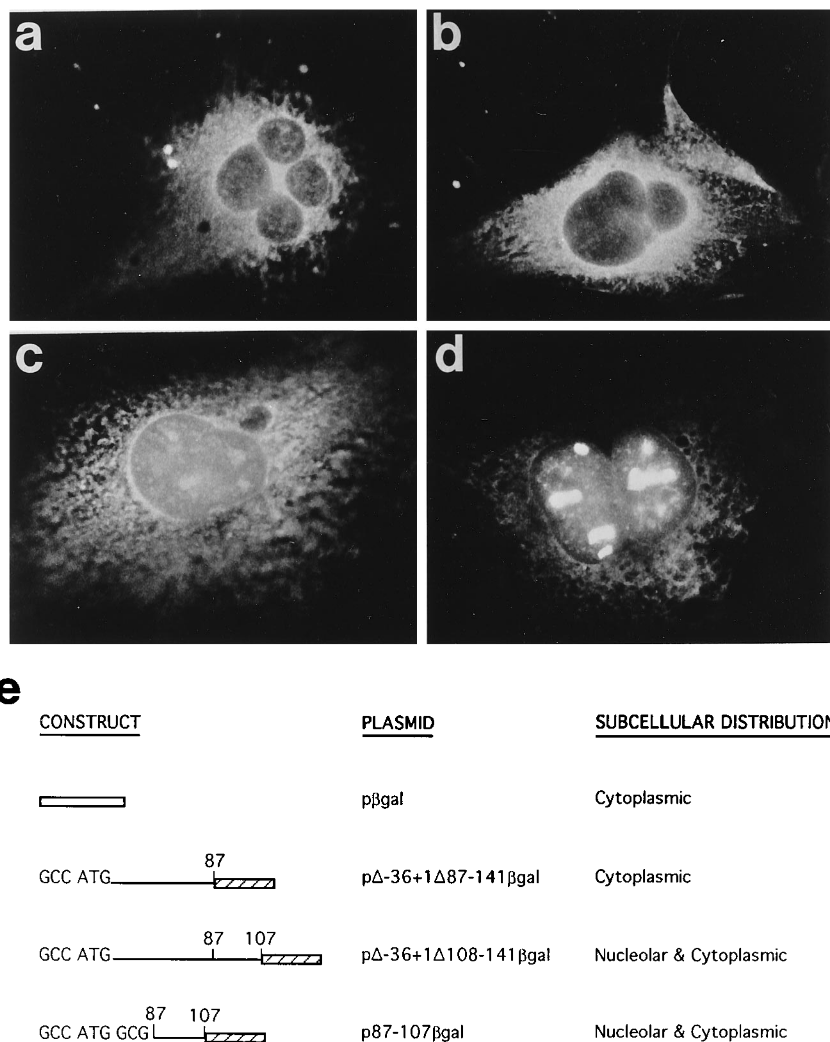

FIG. 4. Immunofluorescence localization of PTHrP/β-galactosidase fusion proteins in COS-7 cells. (a to d) Subcellular localization of transiently expressed PTHrP/β-galactosidase fusion proteins identified by using fractionated ascites fluid developed in mice against *E. coli* β-galactosidase protein. Plasmid transfections were as follows: (a) pβgal, (b) pΔ-36+1Δ87-141βgal, (c) pΔ-36+1Δ108-141βgal, and (d) p87-107βgal. (e) Plasmid constructs and their subcellular distribution. The stippled bar represents the full-length *E. coli lacZ* gene from pCMVβ, and the hatched bars represent the *E. coli lacZ* gene missing the first eight nonessential codons, derived from plasmid pMC-1871. All plasmids were constructed in the pcDNA1 vector. Magnification, ×400.

transfected cells, i.e., predominantly cytoplasmic for the full-length protein (Fig. 8B), predominantly nucleolar for the leaderless protein (Fig. 8C), and exclusively cytoplasmic for the protein missing the NTS (Fig. 8D). Control populations transfected with vector alone showed no specific staining for PTHrP (Fig. 8A).

When cultured in the presence of serum for 24 h, the cell populations appeared similar (Fig. 9a to d). However, morphologic assessment after an extended period of serum deprivation revealed very few adherent cells in control cultures (Fig. 9e) or in those expressing PTHrP without the NTS (Fig. 9h). In contrast, cells expressing preproPTHrP (Fig. 9f) and the nonsecretory mature protein (Fig. 9g), both being forms capable of translocating to the nucleolus, were significantly more resistant to serum deprivation, as evidenced by more adherent cells and fewer refractile bodies. The apparent improved survival of cells expressing the native secretory form (Fig. 9f) perhaps predicts the necessity for entry into the secretory pathway for efficient targeting to the nucleolus to occur. The number of viable, adherent cells remaining at timed intervals following serum withdrawal is shown in Fig. 10. Cell populations expressing protein with an intact signal sequence either increased (pPTHrP/3) or were maintained (pΔ87-107/3) for several days in the absence of serum, whereas populations expressing the mature form of PTHrP (pΔ-36+1/3) or those transfected with vector alone declined soon after serum withdrawal. However, populations expressing protein with no NTS (pPTHrP/3 and pΔ-36+1/3) had an improved long-term survival compared with those transfected with the vector control or those expressing protein with no NTS (pΔ87-107/3). Addition of PTHrP(1-34) at 10^{-9} M had no effect on the survival of control cultures.

DISCUSSION

The results presented in this study demonstrate that a PTHrP sequence, with structural homology to NTSs in human retroviral regulatory proteins, is functional and capable of targeting both intact PTHrP and a heterologous, cytoplasmic protein to the nucleolus. We have also shown, both in vitro and in vivo, that endogenous PTHrP is present in the nucleolus, im-

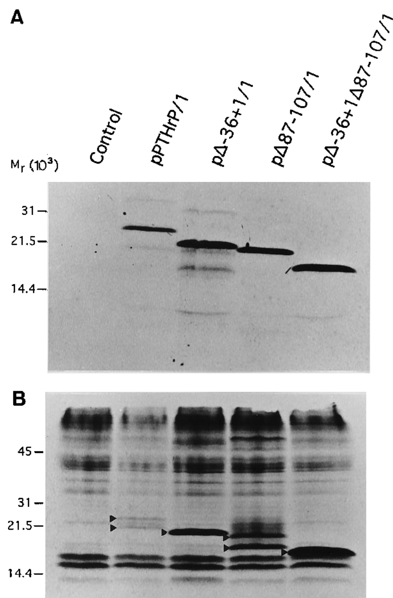

FIG. 5. In vitro translation of PTHrP constructs and immunoprecipitation analysis of transfected COS-7 cells. (A) SDS-PAGE analysis of [^{3}H]leucine-labeled translation products of cRNAs transcribed from the indicated plasmids. Translation was performed in a rabbit reticulocyte lysate cell-free system. Control represents a reaction mixture without cRNA. (B) SDS-PAGE analysis of proteins immunoprecipitated with antiserum raised against PTHrP(1-34), from lysates of cells metabolically labeled with [^{3}H]leucine following transfection with 5 μg of the indicated plasmid DNA. Lysate of cells transfected with pcDNA1 vector was used as control. Approximately 30,000 cpm was loaded into each lane of a 17% polyacrylamide gel. Positions of molecular weight markers run in parallel and stained with silver stain are shown at the left. Arrowheads indicate immunoprecipitated proteins corresponding in size to the in vitro-translated products as well as the appropriately processed peptides.

plying biological significance to this subnuclear localization. Finally, we have presented evidence suggesting that the nucleolar localization of PTHrP may be critical to its role in enhancing survival of chondrocytes in culture under conditions that promote apoptotic cell death.

Localization of peptide growth factors to the nucleolus. Many eukaryotic proteins, including a number of growth-related peptides, have been shown to localize to nucleoli of target cells. This report, however, is the first documenting targeting of a normally secreted, endogenously produced peptide growth factor to nucleoli in vivo. Additionally, this is the first demonstration of a consensus NTS, such as those described for the retroviral regulatory proteins, within a eukaryotic protein. Indeed, localization of most proteins to nucleoli appears to be determined by multiple functional domains rather than by an NTS alone (references 55 and 68 and references therein). The presence of a consensus NTS motif in PTHrP, together with its localization to nucleoli in vivo, is therefore of unusual interest and predicts a physiological role for this peptide within the nuclei of target cells.

Nuclear targeting of PTHrP, or any other secretory protein, could be accomplished by way of two distinct pathways. One would necessitate redirection of the protein away from the classical secretory pathway, while the other would involve endocytosis of the secreted protein. A potential mechanism for redirection could involve the use of alternate splicing, thereby generating mRNA species missing the signal peptide. Although such PTHrP transcripts have not been described to date, this possibility cannot be excluded. An alternate mechanism to bypass the secretory pathway has been described re-

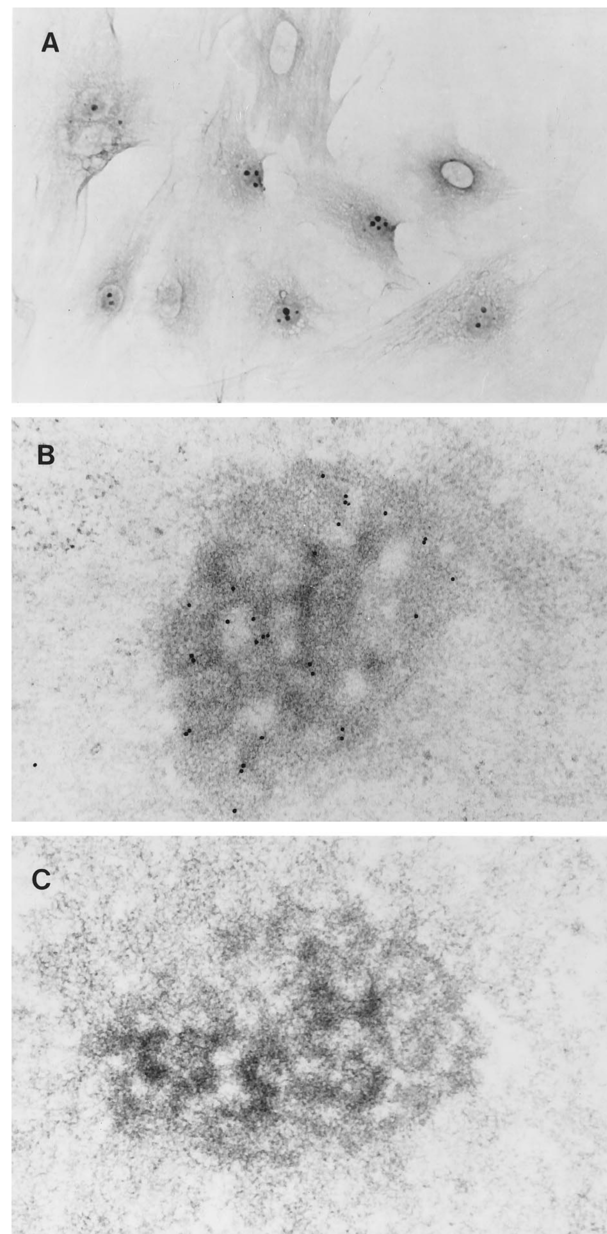

FIG. 6. Immunohistochemical and immunogold localization of endogenous PTHrP in murine bone cells. (A) Osteoblast-like cells harvested from calvariae of 2-day-old mice were maintained in culture and stained immunochemically for PTHrP, using polyclonal antisera directed against various epitopes of PTHrP. Shown is a representative pattern of staining, both nucleolar and cytoplasmic, obtained with rabbit antiserum raised against PTHrP(1-34). Magnification, ×400. (B) Immunogold labeling of an osteoblast nucleolus from a tibia extracted from an 18.5-day-old normal fetal mouse. PTHrP immunoreactivity is observed over the fibrillar component of the nucleolus. No specific labeling was observed in tibial sections from a mutant mouse homozygous for null mutation of the PTHrP gene (C). Magnification, ×27,500.

cently for FGF-3 (34). In this case, a CUG-initiated species was shown to have dual fates, either secreted or targeted to the nucleus, as judged from the competition between secretory and nuclear targeting signals. However, the structural features facilitating this dual targeting in FGF-3 are absent in PTHrP.

Endocytosis of secreted proteins following binding to cell surface receptors and their subsequent translocation to the nuclear compartment have been described for a number of

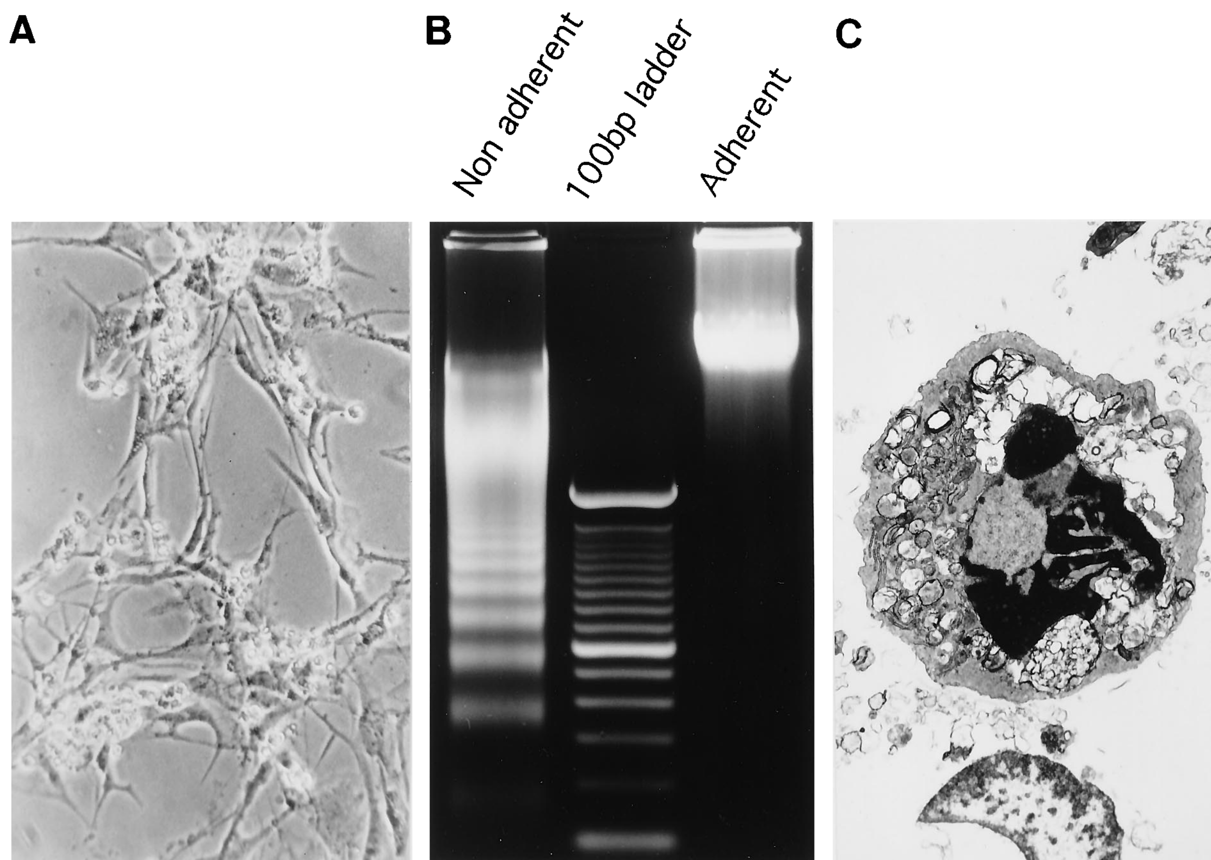

FIG. 7. Serum-deprived CFK2 chondrocytes die by apoptosis. CFK2 chondrocytes stably transfected with pcDNA3 vector were grown in culture medium containing 10% fetal calf serum until 80% confluence and then changed to serum-free conditions. (A) Gross morphologic features of apoptotic cells after 6 days of serum deprivation. (B) Genomic DNA was harvested from both nonadherent and adherent cells after 24 h of serum-free culture for the assessment of internucleosomal fragmentation. Approximately 15 μg of genomic DNA from nonadherent and adherent cells was electrophoresed in a 1.5% agarose gel stained with ethidium bromide, along with a 100-bp molecular size marker. (C) Electron micrograph demonstrating apoptotic features (chromatin condensation, membrane blebbing, and cytoplasmic vacuolization) in an adherent CFK2 cell harvested after 6 days of serum-free culture. Magnification, ×5,000.

growth factors (8, 13, 46) as well as for HIV-1 Tat protein (42). In the case of bFGF, interaction of the peptide with a cell surface heparan sulfate proteoglycan, recently identified as perlecan (6), allows for effective binding of the ligand to its high-affinity tyrosine kinase receptor (9). Receptor-bound bFGF associated with heparan sulfate proteoglycan was subsequently shown to gain access to the cytoplasm following internalization (54). Although well documented, little is known regarding the molecular mechanisms involved in ligand-mediated endocytosis of the G-protein-coupled receptors (45, 58, 65). The receptor for PTH/PTHrP is a member of a newly recognized subfamily of structurally distinct G-protein-coupled receptors which lack the conserved endocytotic motif present in many other family members (57). However, a novel determinant for efficient internalization has recently been identified in the cytoplasmic tail of the PTH/PTHrP receptor (25). Receptor-bound PTHrP could, therefore, enter the cytoplasmic compartment in a manner analogous to that described for bFGF by an as yet unidentified accessory binding protein. Identification of high-capacity, low-affinity binding sites for PTHrP, in association with high-affinity receptors, on squamous carcinoma cells gives credence to this hypothesis (47). Alternatively, endocytosis could be mediated by a receptor distinct from the classic PTH/PTHrP receptor that recognizes the N-terminal domain of PTHrP.

Irrespective of the mechanism by which PTHrP enters the cytoplasm, once it is there, translocation to the nucleolus could occur following effective interaction with a chaperone. We have shown that the PTHrP NTS appears to be both necessary and sufficient for targeting cytoplasmic proteins (mature PTHrP and β-galactosidase) to the nucleolus. This region may, therefore, be recognized by a shuttle protein, such as B23 (12). It has been suggested that B23 translocates the retroviral proteins Rex (3) and Rev (19) from the cytoplasm to the nucleolus following interaction with their NTS motif. Alternatively, accumulation within the nucleolus could be a function of the NTS binding to nucleolar rRNA, as has been postulated for the Tat protein (55).

Presently, it is unclear why only a small percentage of cells in log-phase growth, expressing either transfected full-length preproPTHrP (COS-7 and CFK2) or endogenous protein (osteoblasts), exhibited nucleolar staining. Perhaps translocation of PTHrP to the nucleolus is cell cycle dependent, as is the case for bFGF (8). Approximately 10% of growing bovine aortic endothelial cells demonstrated nucleolar accumulation of exogenously applied bFGF, the translocation occurring in G_1. Perhaps the 10 to 15% of cells with nucleolar PTHrP represent a subpopulation in G_1. Arrest at this stage could prevent an abortive entry into the cell cycle and subsequent death by apoptosis. This would suggest that mechanisms may exist to modulate the timing of its import into the nucleus. Phosphorylation of S/T-P-X-K or S/T-P-K motifs very close to NLSs by

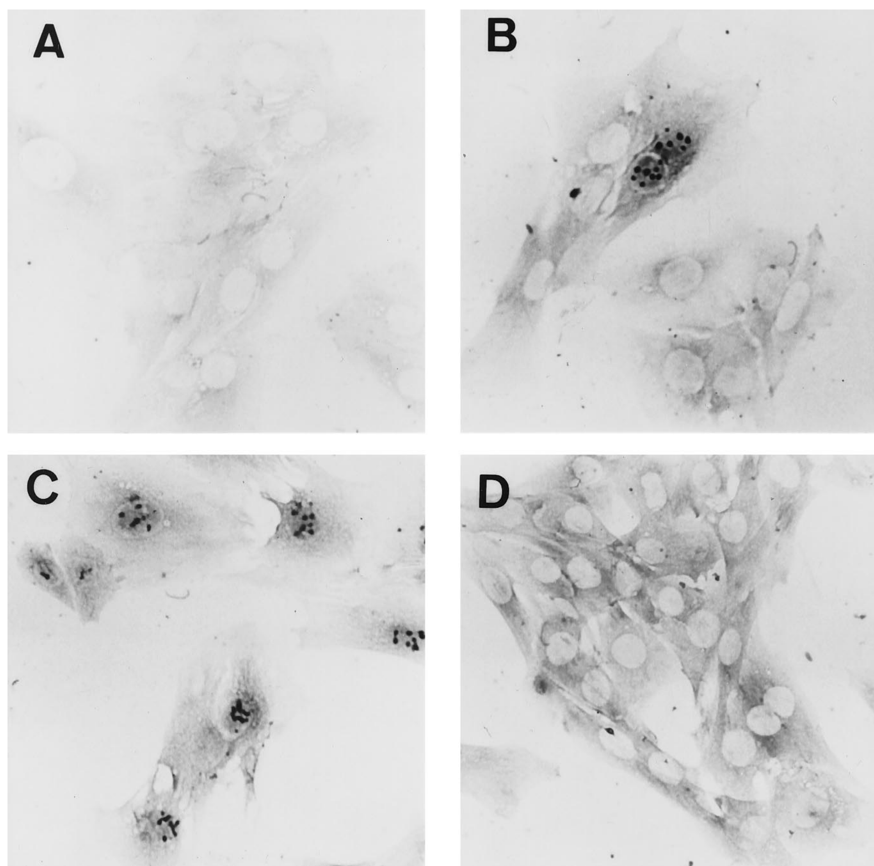

FIG. 8. Immunohistochemical localization of PTHrP in stably transfected populations of CFK2 cells. Populations of CFK2 chondrocytes stably transfected with the various constructs were prepared as described in Materials and Methods. Cells in log-phase growth expressing full-length preproPTHrP (pPTHrP/3) (B), mature PTHrP (pΔ-36+1/3) (C), or preproPTHrP missing the NTS (pΔ87-107/3) (D) were stained immunochemically, using the antiserum raised against PTHrP(1-34). (A) Cells transfected with vector alone (pcDNA3) as a control.

$p34^{cdc2}$ kinase has been described for a number of nuclear proteins such as simian virus 40 T-antigen protein, p53, c-abl, and lamin A/C and may serve as an element of nuclear import regulation (reference 26 and references therein). Phosphorylation at this site in the simian virus 40 T-antigen protein by $p34^{cdc2}$ kinase-cyclin B inhibits its nuclear import (28). A T-85–P–G–K consensus motif for phosphorylation by $p34^{cdc2}$ kinase is located immediately NH_2 terminal to the NTS in PTHrP. Consequently, phosphorylation by $p34^{cdc2}$ kinase may also modulate PTHrP import into the nucleus either by inactivation of the NTS or by induction of cytoplasmic anchoring (28). Alternatively, phosphorylation of PTHrP by $p34^{cdc2}$ kinase, as is the case for other nucleolar proteins, may be important in controlling mitotic changes in nucleolar activity and structure (50).

PTHrP and chondrocyte apoptosis. During embryogenesis and early postnatal life, most chondrocytes undergo further differentiation during the process of endochondral bone formation. Resting chondrocytes first enter an active proliferative phase and then differentiate into large hypertrophic cells. In the zone of vascular invasion, these cells are thought to undergo apoptosis immediately prior to ossification (20) and therefore represent the terminal stage of differentiation in the chondrogenic lineage. Cells that maintain the chondrocyte phenotype, such as those found in the rib, trachea, and articular joints, do not normally undergo hypertrophy and are felt to represent cells blocked at the penultimate stage of chondrocytic differentiation (48). When maintained in vitro, however, these cells will hypertrophy, suggesting that progression to the terminally differentiated phenotype, and subsequent apoptotic death, is prevented in vivo by inhibitory factors.

A critical role for PTHrP in this process was recently suggested by gene targeting studies. Mice homozygous for PTHrP gene ablation display abnormalities in endochondral bone formation, characterized by diminished proliferation and inappropriate differentiation of chondrocytes to the hypertrophic phenotype (4, 32). In view of these observations, PTHrP expression might be expected to delay, or even prevent, the progression to terminal differentiation and eventual programmed cell death. Indeed, preliminary observations indicate that forced expression of PTHrP in CFK2 cells increases their proliferative capacity and inhibits expression of the differentiated phenotype (23). Our current observations in studies using stably transfected CFK2 chondrocytes suggest that PTHrP is capable of prolonging cell survival under conditions that promote apoptotic death. The improved survival results from both the capacity to proliferate in the absence of serum as well as a protective mechanism that involves the NTS. The proliferative component requires the presence of a signal sequence (pPTHrP/3 and pΔ87-107/3), suggesting the necessity for targeting to the secretory pathway. The longer-term protection, however, appears to depend on an intact NTS (pPTHrP/3 and pΔ-36+1/3). The observation that biologically active PTHrP(1-34) added exogenously is incapable of potentiating the prolif-

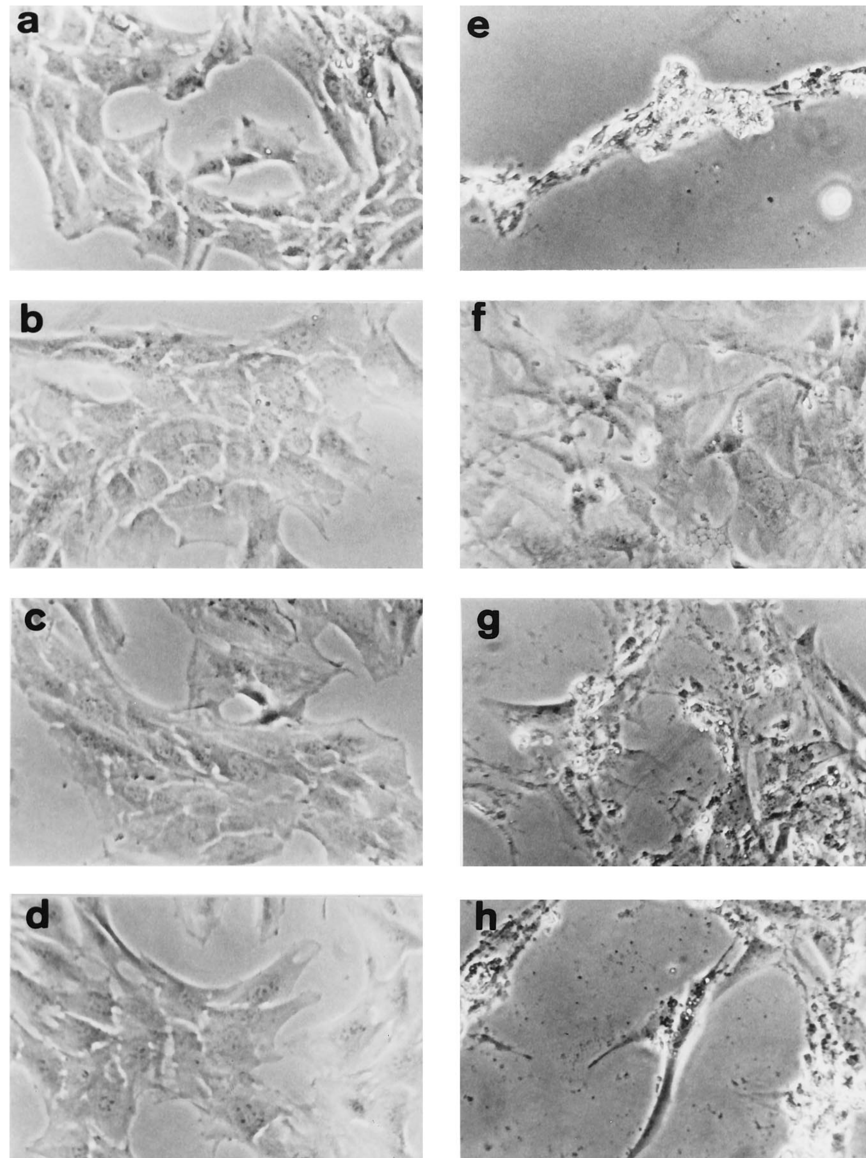

FIG. 9. Stable expression of PTHrP improves survival of serum-deprived CFK2 cells. (a to d) Phase-contrast micrographs of cell populations stably transfected with vector alone (pcDNA3) (a) or expressing full-length preproPTHrP (pPTHrP/3) (b), mature PTHrP (pΔ-36+1/3) (c), and preproPTHrP missing the NTS (pΔ87-107/3) (d) after 24 h of culture in 10% FBS. (e to h) The same cell populations after 9 days of serum-free culture. Magnification, ×250.

eration of control cultures may predict functionality for other parts of the protein.

It should be noted that the HIV-1 Tat protein interacts with a cell surface binding protein through its NTS and is capable of preventing apoptotic death in a number of cell lines following serum withdrawal (71). Moreover, Tat protein has been shown to induce proliferation of human articular chondrocytes primarily through activity of the NTS (37). The presence of a functional NTS in PTHrP and its apparent involvement in the apoptotic process could reflect the presence of a similar mechanism of action for this peptide and explain the diminished proliferative capacity and altered terminal differentiation observed in growth plate chondrocytes from fetal mice homozygous for PTHrP gene ablation.

The precise consequences of PTHrP localization to the nucleolus and how this localization may modulate programmed cell death remain to be defined. The nucleolus is the subnuclear organelle where pre-rRNA is synthesized and ribosomes are assembled. Because of these critical functions, alteration of nucleolar activity may serve as a novel mechanism by which peptide growth factors can modulate the apoptotic process.

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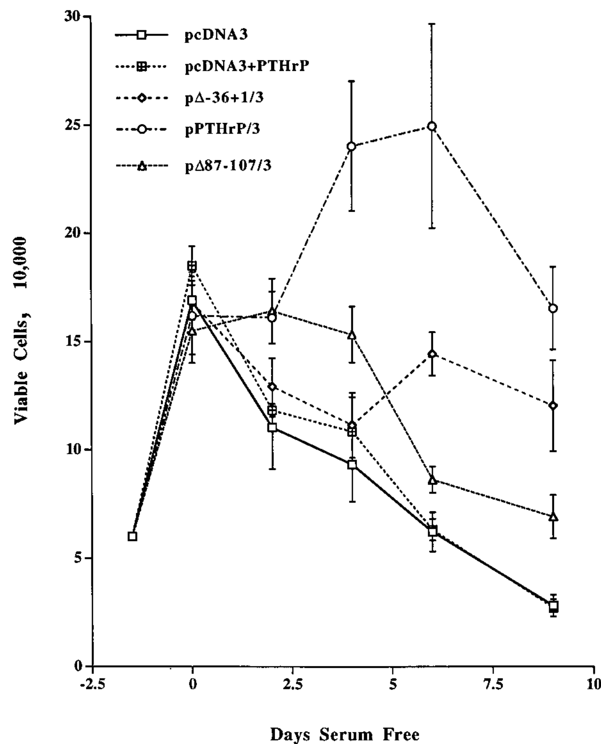

FIG. 10. Survival of stably transfected CFK2 cell populations in serum-free culture. Populations of cells plated at a density of 6×10^4 in six-well plates were deprived of serum after 36 h (day 0). Cells from triplicate wells were counted in the presence of trypan blue at the indicated times following serum deprivation. Each point represents the mean ± standard deviation of four to six individual determinations. Results are representative of three experiments.

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