ABSTRACT A peptide secreted by tumors associated with the clinical syndrome of humoral hypercalcemia of malignancy was recently purified from human renal carcinoma cell line 786-0. The N-terminal amino acid sequence of this peptide has considerable similarity with those of parathyroid hormone (PTH) and of peptides isolated from human breast and lung carcinoma (cell line BEN). In this study we obtained the sequence of a 1595-base cDNA complementary to mRNA encoding the PTH-like peptide produced by 786-0 cells. The cDNA contains an open reading frame encoding a leader sequence of 36 amino acids and a 139-residue peptide, in which 8 of the first 13 residues are identical to the N terminus of PTH. Through the first 828 bases the sequence of this cDNA is identical with one recently isolated from a BEN cDNA library; however, beginning with base 829 the sequences diverge, shortening the open reading frame by 2 amino acids. Differential RNA blot analysis revealed that 786-0 cells express two major PTH-like peptide mRNAs with different 3′ untranslated sequences, one of which hybridizes with the presently described sequence and the other with that reported for the BEN cell PTH-like peptide cDNA. Primer-extension analysis of 786-0 poly(A)+ RNA together with Southern blot analysis of human DNA confirmed the presence of a single-copy gene coding for multiple mRNAs through alternate splicing. In addition, the 3′ untranslated sequence of the cDNA described here has significant similarity to the c-myc protooncogene.

The clinical syndrome of humoral mediated hypercalcemia associated with malignancy (HHM) closely resembles hyperparathyroidism and occurs frequently in patients with certain cancers (1). In 1941, Albright (2) postulated that this syndrome may result from ectopic biosynthesis and secretion of parathyroid hormone (PTH) by tumors. Recent work has revealed that lung (cell line BEN) (3), breast (4), and renal (cell line 786-0) (5) carcinomas secrete peptides similar, but not identical, to PTH. The molecular weight of the lung and breast peptides is ~17,000, and the sequence similarity with PTH was limited to the N terminus (3, 4). A cDNA complementary to the PTH-like peptide (PTH-LP) from a lung carcinoma (cell line BEN) has been cloned and sequenced (6), confirming structural data obtained by polymerase sequencing. Recently a peptide corresponding to residues 1–34 of the PTH-LP was chemically synthesized and confirmed to possess PTH activities on renal and osteoblast membranes in vitro and on calcium and inorganic phosphate fluxes in vivo (7, 8). This structural and functional information has reinforced the hypothesis that HHM is mediated by tumor-secreted factors with biological properties like PTH.

A peptide purified from the human renal carcinoma cell line 786-0 was also found by peptide sequencing to have N-terminal similarity to PTH (9) and to be identical to the N-terminal region of the lung and breast peptides. Like the other PTH-LPs, the renal peptide produced in cell cultures also possesses PTH-like activities. However, the molecular weight of this tumor product was only 6000.

The aim of this study was to characterize the diversity of the PTH-LPs by obtaining the complete deduced amino acid sequence of the renal carcinoma PTH-LP. Using recombinant DNA techniques, we found that the cDNA coding sequence of renal carcinoma PTH-LP was identical to that of the lung-derived PTH-LP cDNA except that it lacks the last two C-terminal residues due to the introduction of a different 3′ untranslated sequence. Our studies indicate that a single gene codes for the PTH-LPs produced by various tumors and that an alternate mRNA splicing mechanism generates multiple PTH-LP mRNAs in 786-0 cells. Furthermore, one of these mRNAs contains similarities to another tumor-associated gene product, the c-myc protooncogene.

MATERIALS AND METHODS

Reagents. Cultures of the human renal carcinoma cell line 786-0 (clone KEC) were maintained as described (9). A cDNA synthesis kit, Hybond-N, [32P]dCTP (3000 Ci/mmol) (1 Ci = 37 GBq) and [γ-32P]ATP (3000 Ci/mmol) were purchased from Amersham. Avian myeloblastosis virus reverse transcriptase was from Seikagaku, KyoGo (Tokyo). Human c-myc exon III DNA was purchased from Oncor (Gaithersburg, MD).

Construction and Screening of a Renal Carcinoma (Cell Line 786-0) cDNA Library. A λgt10 cDNA library was constructed from poly(A)+ RNA prepared from 786-0 cells as described (10). Plaque hybridization was done using a 5′ end-labeled oligonucleotide (5′ CTTCCCCCTTGTCAAGAGAGCTGATGTTCAGACACAGCTC 3′). This sequence is complementary to bases 107–147 of a PTH-LP cDNA reported by Suva et al. (11). Conditions for filter hybridization and washing were 6× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7) at 50°C and 2× SSC/0.1% NaDodSO₄ at 60°C, respectively. Washed filters were exposed to Kodak XAR film using DuPont Cronex intensifying screens.

Subcloning and Sequence Analysis of Clone 10B5. The EcoRI cDNA inserts from positive plaques were subcloned into the EcoRI site of pBS/MI3 + (Stratagene, San Diego).

Abbreviations: HHM, humoral hypercalcemia of malignancy; PTH, parathyroid hormone; PTH-LP, PTH-like peptide.

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†The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03802).
CA), and their sequences were determined by the method of Maxam and Gilbert (11). Sequence analysis was done using IntelliGenetics computer programs.

**RNA Blot Analysis.** Poly(A)+ RNA was prepared from cells or tissue as described (10), electrophoresed in 1% agarose gels containing 2.2 M formaldehyde, and transferred to Hybond-N (12). Blots were prehybridized overnight in a solution containing 50% (vol/vol) formamide at 42°C. Blots were hybridized to cDNA probes at 42°C and washed at 65°C in a solution of 0.1 × SSC/0.1% NaDodSO4. Hybridization of the prehybridization was done at 35°C. The oligonucleotide 10B55' (5'-CGTCCAGGCTTTTGTTTCGTGAAAGGTCTACCGCCTAC-3') was derived from the complement of bases 49–95 of the clone 10B5 sequence. The oligonucleotide AP7 (5'-AGAATCTCTGATGCTCTGGAAAGTGTCGTCTGAAATTATTCATAGGCTCT-3') is complementary to bases 525–576 of the sequence of Suva et al. (6) After hybridization, filters were washed at 60°C in 1 × SSC/0.1% NaDodSO4.

**Primer-Extension Analysis of the 5'-End of Cell Line 786-0 PTH-LP mRNA.** A 25-μmol aliquot of a 5'-end-labeled oligonucleotide 10B55' (specific activity 3 × 107 cpm/μg) was hybridized to 2 μg of cell line 786-0 poly(A)+ RNA as described (13). After hybridizing to primer to RNA overnight at 60°C, primer extension was done for 1 hr at 42°C using 15 units of avian myeloblastositis virus reverse transcriptase. The extension products were separated on an 8% denaturing polyacrylamide gel. Autoradiography of the wet gel was completed with intensifying screens.

**Southern Blot Analysis.** The procedures used in the preparation of large-molecular weight DNA from human term placenta and the subsequent Southern blots have been described (10). The cDNA probes used for Southern analysis are outlined in Fig. 4. Hybridizations and washes were done at 65°C in 6 × SSC and 0.1 × SSC, respectively. A blot prepared using the oligonucleotide AP7 was hybridized at 50°C in 6 × SSC and washed at 65°C in 3 × SSC/0.1% NaDodSO4.

**RESULTS**

**Isolation and Analysis of cDNAs Complementary to the PTH-Like Factor mRNA Expressed in Human Renal Carcinoma 786-0 Cells.** The partial N-terminal amino acid sequence for the PTH-LP secreted by human renal carcinoma cell line 786-0 (6) has at least 50% similarity with PTH and nearly complete similarity to a PTH-LP purified from human lung carcinoma cell line BEN (3). An oligonucleotide encoding the PTH-like sequence of the BEN cell PTH-LP cDNA (see Materials and Methods) was used to screen a λgt10 cDNA library constructed from 786-0 cell poly(A)+ RNA. We isolated three clones varying in size from 625–1600 base pairs in length. The DNA and deduced amino acid sequences of the nearly full-length clone 10B5 are presented in Fig. 1A.

A 303-base 5' untranslated sequence is followed by a 525-base open reading frame. The start codon ATG is preceded by a sequence similar to the consensus sequence for the initiation of translation of eukaryotic mRNA (14). The PTH-like sequence begins at base 412; 8 of the first 15 amino acids encoded are identical to PTH. The remainder of the peptide, as shown by Suva et al. (6), shares only slight similarity to PTH. The nucleotide sequences and open reading frames of the cDNAs for the lung and renal PTH-LPs are identical through base 828, where they totally diverge. In clone 10B5, there is a stop codon, which removes the last two residues (Arg-His) from the BEN cell-derived cDNA sequence. The 3' untranslated sequence from clone 10B5 has 767 bases and is punctuated by two polyadenylation sites (AATAAA) followed by a short poly(A) stretch. In contrast, the 3' untranslated sequence of the BEN cell PTH-LP cDNA is 550 nucleotides in length (6). Interestingly, both 3' sequences are A+T rich and contain multiple copies of an ATTGA motif that has been shown to play a role in mRNA turnover (15).

**Renal Carcinoma Cells Express Two Major PTH-LP mRNAs with Different 3' Untranslated Sequences.** The RNA blot shown in Fig. 2, lane 1, reveals the presence of two major (~1800 and 1550 nucleotides) and one minor (2400 nucleotide) species of PTH-LP mRNAs. This observation could be explained by multiple PTH-LP genes, by the alternate use of different polyadenylation sequences within the 3' untranslated region, or by alternate splicing of different 3' untranslated sequences (16).

To investigate these possibilities, we hybridized 786-0 poly(A)+ RNA to probes derived from the 3' sequences of either 10B5 or the BEN cell cDNA. The 10B5 3'-specific cDNA probe hybridized to the 2400 and 1800 base mRNA (Fig. 2, lane 2), whereas an oligonucleotide (AP7) derived from the 3' end of the BEN cell cDNA hybridized to the 1550-base message (lane 4). Hybridization with both probes (lane 3) demonstrated that all mRNAs were present in the same sample. The blots in lanes 2 and 4 were overexposed to reveal the absence of cross hybridization between the two 3' untranslated sequences. The 5' oligonucleotide 10B55' hybridized to all three mRNAs, indicating that the 5' untranslated sequence of 10B5 is common to all three PTH-LP mRNAs expressed by these cells (lane 5).

To further clarify the basis for multiple mRNAs we have sequenced a cDNA, 10C4, which appears to be derived from a partially processed PTH-LP mRNA. The sequence terminates with a short stretch of adenines, lacks an in-frame stop codon and polyadenylation sites, and diverges at the same point in the sequences as the cell line 10B5 and the BEN cell sequences (BRF.50) cDNAs (6). In Fig. 1B we present a comparison of the three known cDNA sequences derived from this part of the message. From these data we postulate that the point at which the three cDNA sequences diverge is a splice site (17) that can accept one of at least two different 3' untranslated sequences and that the last 35 bases of the cDNA 10C4 sequence are intron sequences.

**Primer-Extension Analysis of the 5' End of PTH-LP mRNA.** Primer-extension analysis was done to investigate whether the multiple PTH-LP mRNAs expressed by 786-0 cells results from initiation of transcription at different promoters (Fig. 3). A 5' oligonucleotide, 10B55', which hybridized to all PTH-LP mRNAs in 786-0 cells (Fig. 2, lane 5), was used as a primer. The two major extension products of 111 and 113 bases in length (Fig. 1A), obtained by using a primer beginning at base 96 of the 10B5 sequence, indicate that the putative 5' cap sites of the PTH-LP mRNAs in 786-0 cells are 15–17 bases upstream from the 5' end of sequence 10B5.

**The PTH-LPs Are Encoded by a Single-Copy Gene in Humans.** Southern blot analysis of human placental DNA was done using the different 32P-labeled cDNA probes outlined in Fig. 4. All probes derived from the 10B5 cDNA (blots A–C) hybridized to a single EcoRI fragment of ~15 kilobases (kb), whereas 5'- or 3'-specific probes hybridized to one of two different Pvu II fragments (blots A and C).

To examine whether the two different 3' untranslated sequences found in the same gene, blot D in Fig. 4 was hybridized to an oligonucleotide (AP7) complementary to the 3' end of the BEN cell PTH-LP cDNA. This sequence hybridized to an EcoRI fragment common to the 10B5 probes (A–C), but hybridized to a unique Pvu II fragment (Fig. 4, blot D). These data indicate that both 3' untranslated oligonucleotide sequences are contained within the same 15-kb EcoRI fragment, but can be mapped to two distinct Pvu II subfragments. To test for possible related sequences within the human genome, an additional blot was hybridized to probe A at lower stringency (55°C). This resulted in a general increase in background
A.

\[
\begin{align*}
&\text{CGGTTCGGAAGAAGCTGACTTCAGAGGGGAAATCTTATTTATTAGGCGGTAGCCCTGTTC} & 70 \\
&\text{CGACCCAGAGGACTGCTGCAGAAGATATCTTGGTTAGACTGGAGCTCAAGCACTACTAT} & 140 \\
&\text{CATGATGATATATAAAAACACTTTTTGCTTTATTTTCCAGAAGGCGCTCGATTTTCTTCT} & 210 \\
&\text{TTCCTTTTTCTTTTCTCGTTGTTGTTTGGAGAAGACCCAGGTTGAGGCGGCTCTAAATA} & 280 \\
&\text{AGTCGAAGGCGGAGGAGGAGCAGTCACTTCTGCGGAGAGGCTGAGGCGGTCGCTTTT} & 350 \\
&\text{AGGCCTGGCGGTCCTAAAGAAGCTTTGGATTGAGTTGATGTCTAGTTTCTGAAAAT} & 420 \\
&\text{AGGTCTGTTGCTGAAATGTTTTTGTCAAGGATATAGGTTTTTCTCATGTATCTTTTTG} & 490 \\
&\text{AGTTCCTTCTCAGAGCCAGAGGAGGAGGTGTCGCTTTTCTCGTTGTTGTTTGGAGA} & 560 \\
&\text{AGGCCTGGCGGTCCTAAAGAAGCTTTGGATTGAGTTGATGTCTAGTTTCTGAAAAT} & 630 \\
&\text{AGGTCTGTTGCTGAAATGTTTTTGTCAAGGATATAGGTTTTTCTCATGTATCTTTTTG} & 700 \\
\end{align*}
\]

![Fig. 1. Nucleotide and deduced amino acid sequences from clone 10B5 cDNA and analysis of the presumptive site of alternate splicing. (A) The open reading frame begins with the AUG at base 304. The amino acid sequence is presented using the single letter designation. Amino acids -1 to -36 represent the presumptive leader plus pro-specific sequence. The vertical arrow designates the start of the 139 residue PTH-LP. These amino acids shared by the PTH-LP and PTH are underlined. Two polyadenylation sites (AATAAA) at the end of the sequence are boldy underlined. (B) These sequences are taken from corresponding regions of three different PTH-LP cDNAs. The nucleotide and deduced amino acid sequences begin with base 817 of the 10B5 sequence or base 514 of the BRF.50 sequence (6). The arrow represents the point at which the three sequences diverge. In-frame stop codons present in the sequences of 10B5 or BRF.50 are underlined. The 10C4 sequence the presumptive intron consensus dinucleotide GT is underlined. The 10C4 sequence terminates with a stretch of adenines.

B.

\[
\begin{align*}
&\text{CTCGATTCCGTAACAGGCTTTCTCAGAGGGGTTGC} & 10B5 \\
&\text{CTCGATTCCGTAACAGGCTTTCTCAGAGGGGTTGC} & \text{BRF.50} \\
&\text{CTCGATTCCGTAACAGGCTTTCTCAGAGGGGTTGC} & \text{10C4 splice} \\
\end{align*}
\]

without the appearance of any new bands (data not shown), indicating the existence of a single gene that encodes multiple 3' untranslated mRNA sequences.

Sequence Similarities Between the 3' Sequence of PTH-LP and c-myc mRNAs. A computer search of the data base of available DNA sequences revealed regions of considerable similarity between the 3' untranslated sequences of sequence 10B5 and the 3' untranslated sequence of the human c-myc protooncogene (18). Several regions of similarity between the two sequences are shown in Fig. 5.

DISCUSSION

We have isolated and sequenced a cDNA that is complementary to the 1800-base mRNA encoding a PTH-LP expressed by the human renal carcinoma cell line 786-0. The predicted N-terminal sequence of this peptide is consistent with the amino acid sequence of the peptide purified from 786-0 cells, and 8 of the 13 amino acids are similar to PTH. This cDNA encodes a molecule of 139 amino acids that is identical to, but two amino acids shorter than, a peptide sequence deduced from a cDNA isolated from a human lung carcinoma (cell line BEN). The lower apparent molecular weight of the peptide purified from the renal carcinoma (6000) compared with the molecular weight of 15,678 deduced from the cDNA may be due to proteolysis during purification. However, we cannot exclude the possibility of cell-specific processing.

Our data clearly show that in the 786-0 cells there are two
major PTH-LP mRNAs, of approximately equal abundance, generated by alternate splicing of sequences at the 3' end of the coding sequence. In addition, our data suggest that the 1550-base mRNA may be the template for the BEN cell-derived PTH-LP cDNA (6), whereas sequence 10B5 is a copy of the 1800-base message. This conclusion is based on equivalent 5' untranslated sequences and the contribution by the poly(A) tail.

Alternate splicing of a single gene, a ubiquitous process in eukaryotic cells, can generate a diversity of protein products and can serve as a potential mechanism for developmental and tissue-specific gene regulation (16). One well-characterized example of this event is the splicing of the calcitonin gene. Tissue-specific alternate splicing of 3' exons of the calcitonin gene results in the synthesis of two structurally and functionally unrelated peptides, calcitonin (18) and calcitonin-gene-related peptide (19).

Breitbart and Nadal-Ginard (20) used a minigene of tropo- nin T to demonstrate that aberrant processing of tropo- nin T mRNA occurs in cells that do not normally express the tropo- nin T message. Their findings suggest that there are cell-specific trans-acting elements responsible for the tissue-specific processing of precursor RNA. For PTH-LP, it is of interest that the human osteosarcoma cell line Saos-2 (data not shown) also expresses approximately equal amounts of the two major PTH-LP messages and that 786-0 cells contain a larger presumptive precursor RNA. Further studies are needed to determine the normal tissue distribution of the PTH-LP messages and the physiological role of alternate splicing.

The mRNAs expressed by the renal carcinoma cells potentially encode two PTH-LPs that would differ by two amino acids at their C termini. Such a minor alteration in the structure of a peptide as a result of alternate splicing is rare (21). The functional consequences in terms of biological activity of such a change is unknown, but this change could

FIG. 2. RNA blot analysis of 786-0 poly(A)+ RNA reveals the presence of two different 3' untranslated sequences in PTH-LP mRNA. A 3-μg aliquot of cell line 786-0 poly(A)+ RNA was separated by agarose gel electrophoresis in the presence of 2.2 M formaldehyde and blotted to nylon membranes. Individual blots were hybridized with the 1595-base-pair 10B5 cDNA (lane 1), bases 872–1595 of clone 10B5 sequence (lane 2), the oligonucleotide AP7 derived from the 3' end of the BEN cell PTH-LP cDNA (6) (lane 4), or the 5' oligonucleotide 10B5s' (lane 5). To generate the blot seen in lane 3, the blot seen in lane 4 was rehybridized to the probe used to generate the blot seen in lane 2 without removing the first probe. RNA size markers are rat 18S and 28S rRNAs.

FIG. 3. Primer-extension analysis of the 5' end of PTH-LP mRNA expressed by 786-0 cells. The end-labeled oligonucleotide 10B5s' (25 pmol) was hybridized to 2 μg of poly(A)+ RNA from 786-0 cells. The extension products were separated by electrophoresis through a 8% polyacrylamide gel containing 8.2 M urea. Autoradiography was done for 2 days. The DNA size markers were a Maxam and Gilbert sequencing ladder (ladder data not shown).

FIG. 4. Southern blot analysis of human placental DNA. Large molecular weight DNA (10 μg) was digested overnight with either Pvu II (P) or EcoRI (E). Digested DNA was electrophoresed through 0.8% agarose gels and transferred to Hybond-N membranes. Individual blots A–D were hybridized to the corresponding DNA probes outlined below. Thin and bold lines indicate unhybridized and coding sequences, respectively. Other restriction sites are Ava I (A), Cla I (C), and HindIII (H). The 52-base oligonucleotide AP7 is derived from the BEN cell PTH-LP cDNA (6). The numbers at the left represent the HindIII-digested DNA size markers and are expressed as the number of kilobase pairs.

FIG. 5. Nucleotide sequence similarity between the 3' untranslated sequences of 10B5 and the c-myc gene. DNA homology search of the GenBank DNA sequence data base and subsequent sequence alignment was completed using the tFIND and ALIGN programs of IntelliGenetics. The asterisks (*) denote base matches between the two sequences, whereas dashes (−) represent gaps introduced to maximize base matching.
potentially have a profound influence on protein secretion. Recently it was reported that a C-terminal tetrapeptide Lys-Asp-Glu-Leu was required for retention of certain proteins in the lumen of the endoplasmic reticulum; the addition of a single amino acid to the end of the protein caused the protein to be secreted (22). Along similar lines, Collins et al. (23) have shown that tissue-specific alternate splicing of platelet-derived growth factor A chain mRNA removes a basic domain at the C terminus that results in a marked decrease in the secretion of the molecule. Alternate splicing of the PTH-LP mRNA may also provide a 3′ untranslated sequence that could influence mRNA stability or translatability.

In contrast to earlier views that the different tumor-derived PTH-LPs may be encoded by a family of genes, the Southern blot analysis indicates that a single-copy gene is responsible for the multiple PTH-LP mRNAs. Suva et al. (6) speculated that different sequences seen at the 5′ end of the cDNA may result from alternate splicing. RNA blot and primer-extension analysis presented here show that in 786-0 cells, the three PTH-LP mRNAs have common 5′ untranslated sequences. The close proximity of the two initiation sites suggests that the two transcripts are initiated by the same promoter. Although it seems unlikely, we cannot exclude the possibility that the gene may encode two 5′ noncoding regions of similar size or that alternate use of 3′ untranslated sequences is determined by the splice site used.

The significance of similarities in the 3′ untranslated sequence of PTH-LP and c-myc mRNAs is unknown at this time. Elevated levels of c-myc have been correlated with cell transformation (24) and may play a role in the phenotype of these tumor cells. The A + U rich sequences shared by the PTH-LP and c-myc messages may be a common signal for mRNA turnover (15). These sequence similarities may be coincidental but are sufficiently interesting to warrant examination of tumor cells that express PTH-LP for a possible regulatory role of the 3′ untranslated regions.

Little is known about the physiological regulation of PTH-LP gene expression. PTH-LP may be the product of a developmentally regulated gene, the transcription of which is promoted as a consequence of cell transformation. Such a phenomenon is exemplified by the ectopic expression of the developmentally regulated c-myc gene in certain hepatomas and teratocarcinomas (25). On the other hand, PTH-LP may normally be expressed at lower levels in nontumor cells, serving a physiological function yet to be elucidated. Further studies are needed to elucidate the pattern of PTH-LP gene expression in normal tissues as well as in tumors.

Note Added in Proof. After the submission of this manuscript a paper by Mangin et al. (26) appeared describing the cDNA of a PTH-like peptide expressed in the human renal carcinoma cell line SKK-1. The coding region of that cDNA sequence is identical to the one presented here; however, the 3′ untranslated sequence is different from this one and is identical to that reported for the cDNA derived from the cell line BEN. In addition, Mangin et al. (26) found that a variety of tumors as well as cultured keratinocytes express multiple PTH-LP mRNAs, indicating that alternative splicing may be a common mechanism responsible for the generation of heterogeneous PTH-LP mRNAs in tumor cells.