MOLECULAR BIOLOGY OF PARATHYROID HORMONE

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I. INTRODUCTION

Maintenance of extracellular concentrations of calcium ion within a narrow range is essential to the well-being and survival of most vertebrates. The prevention of hypocalcemia is particularly crucial since a decrease in calcium ion concentrations results in increased sensitivity of excitable membranes. Uncorrected severe hypocalcemia may result in tetany progressing to convulsions, with death the ultimate outcome in most species. In land dwelling vertebrates, an elaborate endocrine system has developed to protect the organism from hypocalcemia. Parathyroid hormone (PTH), in concert with vitamin D, is the principal hormone that responds to hypocalcemia to increase extracellular calcium levels. PTH and active metabolites of vitamin D cause the net breakdown of the bone, releasing calcium into the extracellular fluid.[1] In the kidney, PTH increases the tubular reabsorption of calcium, thereby decreasing the excretion and loss of calcium into the urine.[1] PTH also indirectly stimulates the absorption of calcium from the intestine through effects on the renal metabolism of 25(OH) Vitamin D_3.[2] The effects of PTH on the bone and the kidney are important for the short-term regulation of extracellular calcium concentrations. The effects on the kidney, and indirectly on the intestine, are also important for the long-term regulation of calcium balance.

The activity of the parathyroid gland is regulated primarily by extracellular concentrations of calcium ion. As calcium concentrations decline from the normal 2.5 m*M* into the hypocalcemic range, the secretion of PTH is stimulated rapidly.[3,4] A maximal change in secretion rate of about fivefold is observed with a change in extracellular calcium concentrations of only about 1 m*M*, both in vivo and in vitro. In contrast to the rapid, large change in secretion of PTH, PTH synthesis is not greatly affected by acute changes in extracellular calcium concentrations.[5] Chronic hypocalcemia, however, does lead to hypertrophy of the parathyroid gland and increased production of PTH.[1]

The biosynthetic pathway of PTH has been studied intensively in the last decade. The intracellular processing of PTH has been described and involves the synthesis of an initial translational product, preProPTH, and two proteolytic cleavages that in turn produce ProPTH and PTH. The mRNA of the bovine PTH has been characterized, cDNAs and genes for PTH mRNA have been cloned and sequenced, and the chromosomal site of the human PTH gene has been localized. The purpose of this review is to summarize these advances in the molecular biology of PTH, from the gene to the cotranslational processing of the initial translational product, preProPTH. Discussion of the posttranslational events, including intracellular proteolytic processing of ProPTH and PTH, secretion of PTH, metabolism of PTH in the circulation, and its mode of action may be found in recent comprehensive reviews.[5-7]

II. PREPROPTH

The primary form of PTH, which is stored in the gland and secreted, contains 84 amino acids.[8] Biosynthetic studies in intact cells demonstrated that PTH was initially synthesized as a precursor, ProPTH, that contained six extra amino acids at the N-terminus.[9,10] Conversion of ProPTH to PTH occurred about 15 to 20 min after biosynthesis at about the time ProPTH reached the Golgi apparatus.[11]

A. Structure of the Pre-Peptide

Evidence that the translational product of PTH mRNA was larger than ProPTH was initially obtained by translation of a crude preparation of bovine parathyroid RNA in the wheat germ cell-free system.[12] The primary translational product migrated slower than ProPTH when analyzed by electrophoresis on either acidic-urea or sodium dodecyl sulfate-containing acrylamide gels. At that time, a similar phenomenon had been observed only for myeloma light chains.[13] Early evidence that the larger product was not an artifact of the wheat germ cell-free system included the demonstration that the N-terminal amino acid was methionine and was contributed by the initiator methionyl mRNA.[14] In addition, the two proline containing tryptic peptides of PTH, one of which was the C-terminal peptide, had identical mobilities with those of preProPTH when analyzed by paper chromatography or electrophoresis.[14] In further studies, preProPTH was shown to be synthesized in cell-free systems based on Krebs ascites[15] and reticulocyte lysates.[16] Translation of human parathyroid RNA also produced an analogous preProPTH.[17]

The observation that the carboxyl terminal peptides of bovine PTH and preProPTH were identical indicated that the extra amino acids in preProPTH were at the amino terminus. This was confirmed by incorporating selected radioactive amino acids into preProPTH and determining the location of the radioactivity by automated Edman degradation.[18] By analyzing overlap of these radioactive amino acids with those in ProPTH, the length of the bovine pre-peptide was shown to be 25 amino acids. The entire sequence of the bovine pre-peptide was determined eventually by this microsequencing technique[19] and was later confirmed by structural studies of both the bovine PTH cDNA and gene.[20-22] The sequence of human pre-peptide was also partially determined by this microsequencing technique.[17] The complete amino acid sequence was derived from the human PTH cDNA sequence[23] and later confirmed by the determination of the structure of the human gene.[24] The amino acid sequence of the rat pre-peptide has recently been derived from the sequence of the rat PTH gene[25] and partially by analysis of cloned rat PTH cDNA.[26]

The amino acid sequences and predicted secondary structures of the pre-peptides are shown in Figure 1. The human and bovine pre-peptides are 80% homologous while the rat sequence is 64% homologous to the bovine and human. This is somewhat lower than the homology of 89 and 77% in the Pro and PTH regions for bovine/human and rat/bovine-human, respectively. The fact that the pre-peptide is less conserved than the rest of the molecule is consistent with the observations that little discernible homology is present when the known pre-peptides or signal peptides of many eukaryotic proteins are compared.[27] General structural features of the signal peptides are a central hydrophobic core and, in many cases, charged amino acids at the N-terminal and C-terminal ends of the central core. These features are largely retained in the pre-peptides of the three preProPTH molecules (Figure 1). Only conservative changes are present within the central core of uncharged amino acids from amino acids 10 to 21. In the rat, changes at positions 5, 6, and 22 result in the loss of two positive charges and one negative charge. However, a single positively charged amino acid before and a single negatively charged amino acid after the hydrophobic core are retained in the rat sequence.

The secondary structure of the pre-peptide of bovine PTH predicted by the Chou-Fasman method[28] contains a β turn for four amino acids at the cleavage site (Figure 1, Table 1),[29,30]

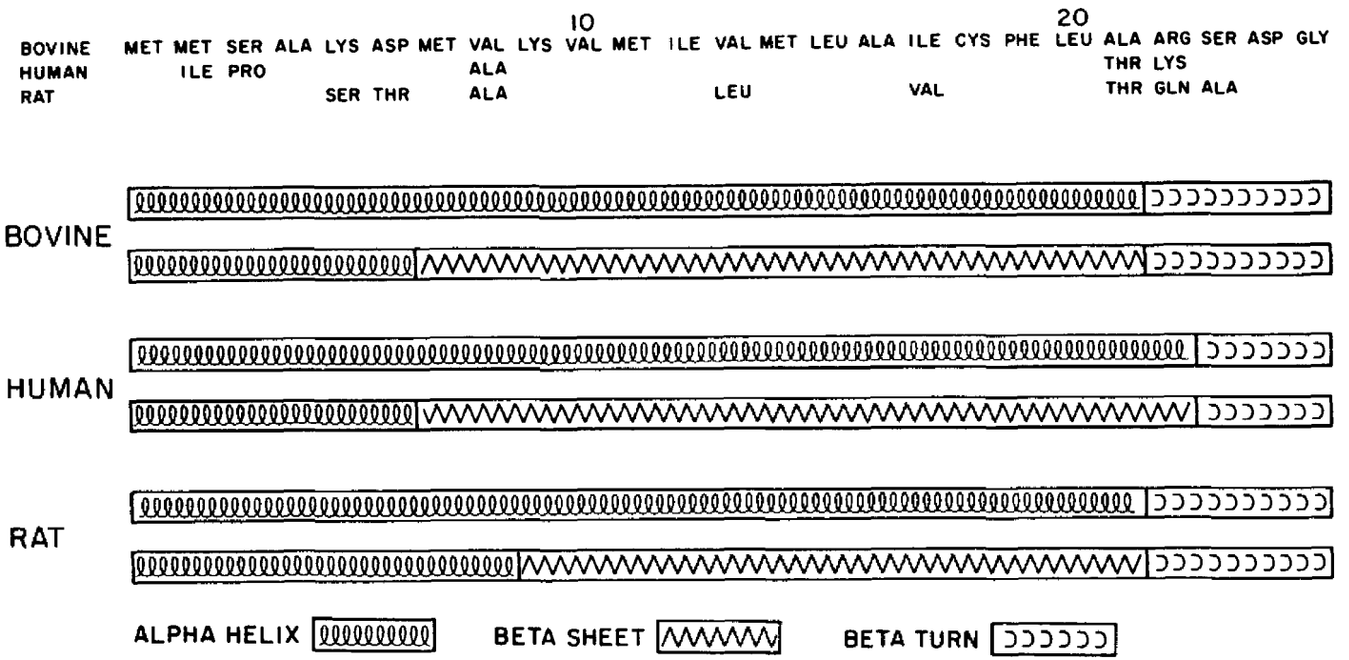

FIGURE 1. Comparison of the amino acid sequences and predicted secondary structure of the pre-peptides of bovine, human, and rat preProPTH. The predicted secondary structure was determined by the method of Chou and Fasman[28] and the average propensities for each of the secondary structures is given in Table 1. Each sequence has two high probability predicted sequences, which either contains predominantly α helix or β sheet.

Table 1
AVERAGE PROPENSITIES FOR SECONDARY STRUCTURE OF BOVINE, HUMAN, AND RAT PRESEQUENCES

Bovine				Human				Rat			
Sequence	Pa*	Pb	Pt	Sequence	Pa	Pb	Pt	Sequence	Pa	Pb	Pt
1—24	1.165	1.092		1—24	1.139	1.077		1—24	1.173	1.092	
7—21	1.196	1.268		7—21	1.181	1.234		9—15	1.231	1.249	
22—25	0.833	0.743	1.350	23—26	0.878	0.695	1.365	10—23	1.173	1.246	
								22—25	1.028	0.805	1.165

* "P_x" Is average propensity for X = a, alpha helix, X = b, beta sheet, and X = t, beta turn.

a feature shared by most other pre-peptides.[31] The remainder of the sequence has nearly equal probability of being in a α helix (1 to 21), or a combination of α helix (1 to 6) and β sheet (7 to 21). These predicted secondary structures for the human and rat pre-peptides are very similar to that of the bovine pre-preptide. Thus, in spite of the fact that 8 of 25 amino acids are changed including replacement of three charged amino acids in the rat compared to bovine sequence, the secondary structure appears to be conserved.

Analysis of the circular dichroism spectra of a synthetic bovine prePro sequence was consistent with a high β sheet structure in an aqueous environment and a high α helical structure in a nonpolar environment,[30] in agreement with the Chou-Fasman predictions of two high probability states. This observation raises the intriguing possibility that the penetration of the pre-peptide into the nonaqueous environment of the membrane may cause a conformational change that is important in its function as a signal sequence.

B. Conversion of PreProPTH to ProPTH

The removal of the pre-peptide to produce ProPTH is mediated by an enzyme associated with microsomal membranes.[16] In the reticulocyte and wheat germ systems that contain little or no microsomal membranes, the primary translational product of PTH mRNA is pre-ProPTH.[12,16] In contrast, in Krebs II ascites cell-free extracts, which contain some microsomal membranes, both preProPTH and ProPTH are produced.[15] Addition of microsomal membranes from dog pancreas or chicken oviduct results in the synthesis of ProPTH.[16,32] In addition, ProPTH, but not preProPTH, is protected from exogenously added proteolytic enzymes, indicating that ProPTH has been transported across the membranes of the microsomal vesicles.[16] If membranes are added after the synthesis of preProPTH is completed, no conversion of preProPTH to ProPTH is observed, nor is the preProPTH protected from exogenous proteolytic enzymes. In these cell-free systems, ribosomes associated with radioactive mRNA become bound to membranes and are resistant to dissociation by high salt as are membrane bound ribosomes formed in vivo.[33] The pre-peptide thus appears to function as a signal peptide as described in the model proposed by Blobel and Sabatini,[34,35] and the signal mechanism is neither tissue nor species specific. Although not yet demonstrated directly for preProPTH, the process presumably involves the interaction of the pre-peptide on the nascent chain with the signal recognition particle and binding of this complex to the signal recognition particle receptor on the endoplasmic reticulum membrane. This is followed by vectorial transport of preProPTH across the membrane.

The first direct evidence that pre-peptides or signal peptides actually function by binding to a limited number of sites in the microsomal membrane was obtained by studies on a synthetic prePro-peptide of bovine preProPTH.[36] The addition of the synthetic prePro-peptide to reticulocyte cell-free systems containing microsomal membranes inhibited the processing of bovine preProPTH, pregrowth hormone and preprolactin, and human preplacental lactogen. As a control, a hydrophobic internal peptide of PTH was added that had no effect on the processing. This inhibition supports the idea that the pre-peptide interacts with a limited number of sites and, thus, that initial interaction with the microsomal membrane is receptor mediated, an idea that has been questioned.[37-39] The recent identification of the signal recognition particle as the signal peptide receptor has conclusively confirmed this mechanism for most secreted and membrane proteins.[40]

The pre-peptide of preProPTH is apparently rapidly degraded after its proteolytic cleavage from preProPTH. Even though five of the seven methionines in preProPTH are present in the pre-peptide, ^{35}S-labeled pre-peptide cannot be detected on gels, while ProPTH formed in cell-free systems is easily detected.[16] In studies of PTH biosynthesis in intact cells, no labeled pre-peptide could be detected, even though labeled prepro-peptide added exogenously to parathyroid extract could be detected in good yield.[41]

The proteolytic removal of the pre-peptide probably occurs before completion of the

prePro PTH nascent chain, since prePro PTH is difficult to detect in intact cells. PrePro PTH that is detected in slices of parathyroid tissue incubated in vitro[42,43] probably represents aberrant synthesis, since Pro PTH is detected before the prePro PTH.[43] The aberrant synthesis of prePro PTH may be related to its relatively short length. Preproinsulin, which is also relatively small, has been detected in intact cells.[44] Presumably, the synthesis of some prePro PTH molecules may be completed before interactions with the signal recognition particle occurs. Since the signal recognition particle will not recognize prePro PTH post-translationally,[16] these prePro PTH molecules will remain in the cytoplasm and be destroyed. In support of this idea, the processing of prePro PTH in in vitro systems is fairly inefficient compared to many other preproteins.[16] A "stretched" version of prePro PTH containing 34 extra amino acids in an internal duplication is processed more efficiently than prePro PTH itself in wheat germ cell-free systems.[32]

C. Analysis of Structure-Activity Relationships

A number of questions remain unanswered about the PTH signal peptide. The relationship of the structure of signal peptides to their function is presently being studied by several groups. Little homology is retained in the primary structure of signal peptides yet most, if not all of them, interact with a signal recognition particle that has been strongly conserved in structure.[35,45] The secondary structure of the pre-peptides, which has been conserved, is probably crucial to its interaction with the signal recognition particle. In addition, while the signal peptide is generally equated with the pre-peptide that is removed from preproteins, it is possible that signal activity involves additional sequence, and cleavage simply inactivates the signal function. This possibility is particularly interesting in the case of proteins, like PTH, that have a pro-sequence of unknown function following the presequence.

To study structure activity relationships of the bovine PTH pre-peptide, we have constructed a plasmid containing PTH cDNA immediately adjacent to a promoter for the Salmonella virus SP6 RNA polymerase.[32] This plasmid also contains a DNA fragment from an f1 virus that allows the plasmid to replicate as single stranded DNA when the bacteria are coinfected with a helper f1 virus. This vector system should permit rapid introduction and characterization of mutations in the signal sequence coding region by methods applicable to either double-stranded or single-stranded DNA. In order to analyze the signal sequence of prePro PTH in the reticulocyte cell-free system, which is more efficient than the wheat germ system, we have constructed a cDNA that codes for a "stretched" prePro PTH. This was necessary because the prePro PTH and Pro PTH cannot be resolved after electrophoresis on gels containing sodium dodecyl sulfate because they migrate near the massive amounts of globin in the reticulocyte lysate. The stretched prePro PTH, which contains a 34 amino acid internal duplication, can be efficiently synthesized and processed in the reticulocyte cell-free systems. In vitro mutagenesis combined with this system should permit a systematic study of the structure-functional relationships of the signal sequence. Similar cell free studies on human prePro PTH using *Escherichia coli* polymerase to synthesize prePro PTH mRNA are also being done.[93]

An alternate approach to these cell-free studies is the introduction and expression of mutated and normal PTH cDNA into heterologous cells. Human PTH cDNA has been inserted into a retrovirus vector followed by infection of rat pituitary GH_4 cells.[46] Pro PTH was synthesized and secreted by these cells even though they do not normally secrete PTH. Analysis of mutated PTH cDNA in cells will complement the cell-free studies and also allow examination of steps in the secretory pathway beyond the initial interaction of the signal peptide with the microsomal membrane. These types of experiments should soon provide a better understanding of the structural requirements for PTH signal function.

III. PTH mRNA

Bovine PTH mRNA has been more extensively characterized than the mRNAs from the other species. Preparations of bovine parathyroid RNA were obtained that contained about 50% PTH mRNA as estimated by gel electrophoresis and RNA excess hybridization to radioactive cDNA.[47] The size of the mRNA was estimated to be about 750 nucleotides by sucrose gradient centrifugation. About two thirds of the translatably active mRNA was retained by oligo(dT) cellulose, and the sizes of the poly(A) extension was broadly distributed around an average size of 60 adenylyate residues. While not directly determined, PTH mRNA probably contains a 7-methylguanosine cap since the translation of PTH mRNA was inhibited by 7-methylguanosine-5'-phosphate.[48] The human and bovine PTH mRNAs appear to be heterogeneous at the 5' terminus (see section on genes). The sizes of the rat and human PTH mRNAs have been determined by Northern blot analysis to be about 800 and 850 nucleotides, respectively.[23,25] PTH mRNAs, thus, are typical eukaryotic mRNAs that contain a 7-methyguanosine cap at the 5' terminus, polyadenylic acid at the 3' terminus, and are larger than necessary to code for the primary translational product, in this case greater than twice as large.

IV. PTH cDNA

A. Cloning of cDNAs

DNA complementary to bovine[20,21] and human[23] PTH mRNA has been cloned into the Pst 1 site of pBR322 by the homopolymer extension technique,[48] and the rat PTH cDNA[26] has been cloned by the Okayama and Berg[49] method. The bovine mRNA was isolated from normal parathyroid glands, and the human mRNA was isolated from parathyroid adenomas. Essentially, the complete sequence of the mRNAs were derived from the cloned cDNAs. The sequence of the rat mRNA has been derived partially from the rat cDNA and from the sequence of the cloned gene.[25]

Kronenberg et al.[20] initially determined the sequence of a bovine cDNA clone, pPTHm1, that contained about 60% of the PTH mRNA, including all of the region coding for pre-ProPTH. Restriction analysis of near full-length double-stranded cDNA, synthesized enzymatically from partially purified bovine PTH mRNA, indicated that about 200 nucleotides from the 3' untranslated region were missing in the clone.[50] A near full-length clone of bovine PTH mRNA, pPTHi4, was sequenced later.[21] The overlapping sequences of pPTHm1 and pPTHi4 were identical except for the first 50 nucleotides of each. Analysis of several additional bovine PTH cDNA clones and the sequencing of cDNA of the 5' terminus of PTH mRNA, which was synthesized by extension of a primer with reverse transcriptase, indicated that the sequence in pPTHi4 was correct.[51] The first 53 nucleotides in pPTHm1 were the reverse complement of the expected sequences. One of the additional bovine clones analyzed, pPTHi8, had 40 extra nucleotides at the 5' terminus that did not correspond to the mRNA. This sequence was an inverted repeat of a sequence about 200 nucleotides from the 5' end of the mRNA. Likewise, a human cDNA clone contained an inverted repeat of an internal sequence.[23] In each of these cases, the 3' terminus of the repeat (relative to the mRNA), was complementary to another internal sequence in the mRNA. This suggests that the single strand of the cDNA folds back on itself either during the reverse transcription or the synthesis of the second strand by DNA polymerase to produce the inverted sequence.[51]

The 5' end of the bovine mRNA sequence, which was not represented in the cloned cDNAs, was determined by sequencing DNA complementary to the 5' end of PTH mRNA produced by primed reverse transcription.[51] Multiple 5' termini of the mRNA were observed by this technique. The heterogeneity at the beginning of the 5' end of the mRNA was confirmed by S1 nuclease mapping.[22] The longest primed reverse transcript was isolated

Table 2
COMPARISON OF THE SIZES OF BOVINE, HUMAN, AND RAT PTH mRNA

	Total nucleotides	5′ Untranslated	Translated	3′ Untranslated
Bovine	701/672[a]	129/100	345	227
Human	822/793	129/100	345	348
Rat	712	132	345	239

[a] The bovine and human genes contain two TATA sequences and probably two functional promoters about 30 nucleotides apart. The two sizes of mRNA represent initiation of RNA synthesis at the sites directed by the two promoters. The rat gene has only one TATA sequence that corresponds to the upstream TATA sequences in bovine and human.

and sequenced. Surprisingly, this cDNA contained a canonical TATA sequence at the beginning, which was in the proper position to direct the transcription of the shorter mRNAs. This result suggested that a second TATA sequence would be present 5′ of the one detected in the cDNA and would direct the synthesis of the longer mRNAs. The predicted second TATA sequence was discovered when the gene was sequenced. The 5′ end of the rat PTH mRNA was also analyzed by S1 nuclease mapping and was less heterogeneous than the bovine mRNA.[25] The single species of rat PTH mRNA corresponded to the larger of the bovine mRNAs. The size of the human mRNA, based on the cDNA sequence, is about 100 nucleotides longer than the bovine and rat mRNAs (Table 2). Northern blot analysis of the mRNAs was consistent with these predicted sizes.[25] The difference in size primarily results from the difference in the size of the 3′ untranslated region.

B. Homology of Sequences

The nucleotide sequences of the PTH cDNAs and the amino acid sequences of the preProPTHs are compared in Figure 2. Gaps have been introduced in the 5′ and 3′ untranslated regions to maximize homology. The overall homology of the bovine to human cDNA is about 70%, and the homology of the rat sequence to the bovine or human sequences is 58 and 61%, respectively, if each position in a gap is counted as a mismatch (Table 3). Alternatively, if gaps of five or more nucleotides are assumed to result from a single mutation event, and are counted as a single difference, the homologies are 85% for bovine to human and 69 and 76% for rat to either bovine or human, respectively. As expected, the coding regions of the sequences have been most conserved with the human and bovine sequences being 90% identical and the rat 77 to 78% homologous to bovine and human. The 5′ noncoding region is also well conserved with homologies about 15% less than the coding region. The 3′ noncoding regions appear most dissimilar. This region in the human sequence is more than 100 nucleotides longer than the other two sequences. Therefore, large gaps have to be introduced into the bovine and rat sequences to maximize homology. In spite of the apparent lack of homology, the nucleotides are conserved as well as or better than in the 5′ noncoding region. Hendy et al.[23] suggested that the extra sequence in the 3′ region of the human cDNA, corresponding to the large gap in the bovine sequence, might have been the result of a gene duplication since it contained some homology to the region around the polyadenylation signal, including a second consensus polyadenylation signal. Interestingly, in the rat sequence, large gaps also must be introduced in this region, but they do not coincide exactly with that of the bovine sequence. Thus compared to the human sequence, 16 of 17 nucleotides in the rat are identical at the beginning, and 16 of 22 nucleotides are identical in the middle of the large bovine gap. This could be explained by separate early gene duplication events in both the rat and human genes. An alternate and simpler explanation would be that the ancesteral PTH gene underwent a duplication in this region followed by

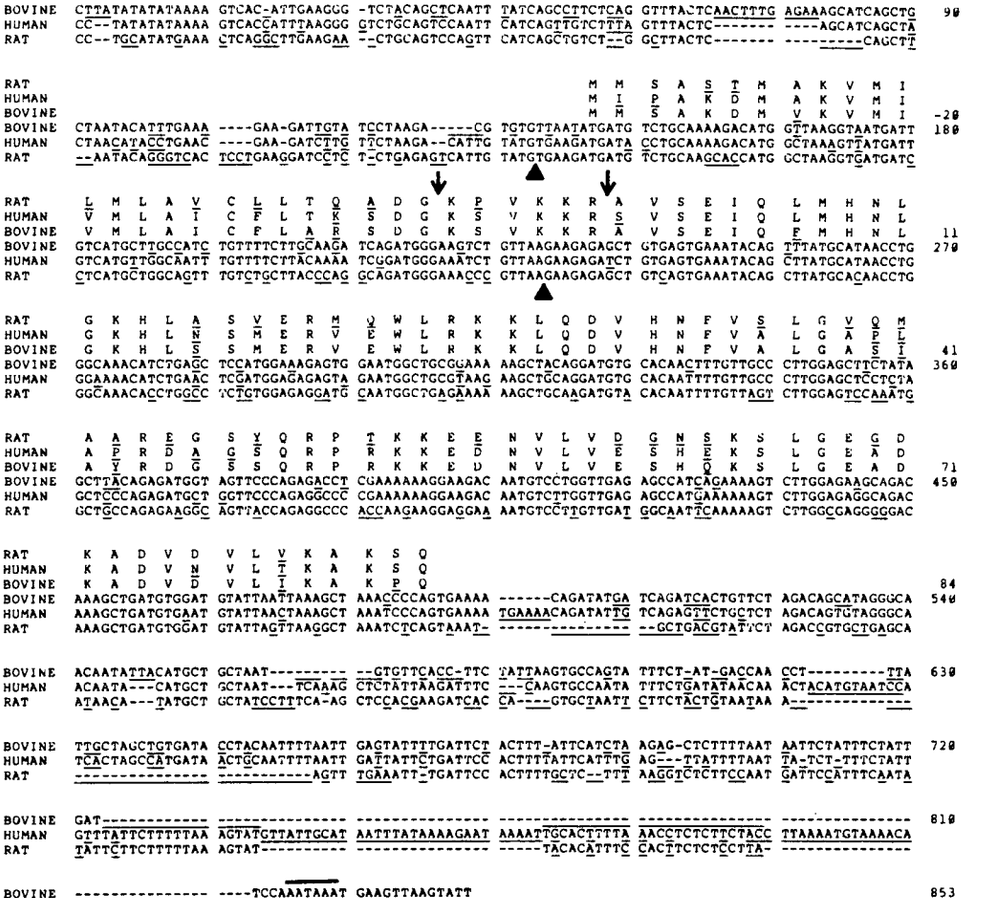

FIGURE 2. Comparison of the nucleotide sequences of the bovine, human, and rat PTH cDNAs and their respective amino acid sequences. Nucleotides or amino acids in one sequence that are different from both of those in the other two sequences are underlined. Gaps indicated by dashes were introduced to maximize homology in the 5′ and 3′ untranslated regions. The arrows indicate the sites of proteolytic cleavage required for the conversion of preProPTH to ProPTH and ProPTH to PTH. The closed triangles indicate the positions of the two introns in the gene. The polyadenylation signal is overlined. The rat sequence shown is derived from the rat gene sequence reported by Heinrich et al.[25] The rat cDNA reported by Schmelzer et al.[26] differs at six nucleotides as follows: G(197) = A, G(211) = A, A(213) = G, G(241) = A, T(329) = G, and A(330) = T. These differences also result in four amino acid differences as follows: cys(−14) = tyr, ala(−9) = thr, val(2) = ile and val(31) = gly.

varying extents of deletion. In the latter case, the duplicated regions would probably be more susceptible to unequal crossing over events, increasing the probability of the deletion of sequence.

The overall nucleotide compositions of the three cDNAs are similar. All the sequences are A-T rich. The 3′ noncoding region has a particularly large portion of A and T, ranging from 68 to 74%. The rat sequence differs from the other sequences in that the 5′ noncoding region is only 50% A and T compared to 63 to 65% for the human and bovine. Analysis of dinucleotide frequencies indicated that with the exception of CG, the frequencies were approximately as expected on the basis of overall nucleotide composition. The frequency of CG was less than 20% of the expected value, which is a common feature of eukaryotic

Table 3
SIMILARITY OF THE NUCLEOTIDE SEQUENCES OF BOVINE, HUMAN, AND RAT PTH GENES

Region	Bovine/human	Bovine/rat % Identical nucleotides	Human/rat
5′ Untranslated	70(77)*	55(62)	69(71)
Translated	90	77	78
3′ Untranslated	52(85)	37(63)	42(75)
Total cDNA	70(85)	58(69)	61(76)
5′ Flanking	74(77)	52(55)	64
Intron A	65(76)	53(55)	47(57)
Intron B	65(74)	37(49)	48(68)
3′ Flanking	76	28	28

* Numbers in parentheses are the percent similarity when the gaps of more than five nucleotides were considered a single mismatch. Otherwise, each nucleotide position in the gap was considered a mismatch.

genes.[52] Since CG is a site of methylation and potentially a mutational "hotspot", selection for the elimination of this dinucleotide in the region of functional genes is likely.[53]

C. 5′ Untranslated Region

The 5′ untranslated sequence is unusually long compared to most eukaryotic mRNAs.[54] The longer forms of the human and bovine mRNAs and rat PTH mRNA contains about 120 nucleotides, and the shorter bovine and human cDNAs contain about 100 nucleotides in the 5′ noncoding region. Of eukaryotic mRNAs, 70% contain less than 80 nucleotides in their 5′ noncoding region.[54] While most mRNAs with long 5′ noncoding regions have specialized function, such as those for heat shock proteins, there is no obvious reason why PTH mRNAs should. Because of the long 5′ noncoding sequence, the m^7G cap at the 5′ terminus is a considerable distance from the initiator codon. In the bovine sequence, a possible hairpin loop might bring the 5′ end closer to the initiator codon. However, in both the human and rat sequences, deletions of 11 and 16 nucleotides, respectively, beginning at nucleotide 69 in Figure 2 largely eliminate the sequences involved in the stem of the loop. Thus, there seems to be little functional significance related to the bovine secondary structure. The sequence $CTTCTC_{56}$ in the bovine sequence is similar to the sequence CTTPyTC found in many globin and other mRNAs,[55] but again this region is not conserved in the other two sequences. Likewise, regions that might hybridize to the 18S rRNA and promote initiation in a manner analogous to prokaryotic systems, are present in the bovine PTH mRNA, but not in the other sequences. The most outstanding conclusion from a comparative analysis of the three mammalian sequences is that no region in the 5′ untranslated region is conserved that has any known functional significance.

D. Coding Region

The actual initiator ATG codons for the human and bovine have been identified by sequencing in vitro translation products of the mRNAs.[17,18] In the bovine sequence, the first ATG codon is the initiator codon, in accord with greater than 90% of other eukaryotic mRNAs.[54] The initial report that an ATG was present in the 5′ noncoding region[20] was the result of a cloning artifact as discussed above. Ironically, the human and rat sequences do have ATG triplets prior to the probable initiator ATG, which are present ten nucleotides before the initiation codon and are immediately followed by a termination codon. In the rat, another ATG is present 115 nucleotides before the initiator codon. The designation of the

Table 4
CODON USAGE OF BOVINE (B), HUMAN (H), AND RAT (R) PARATHYROID HORMONE GENES

		H	B	R			H	B	R			H	B	R			H	B	R
Phe	TTT	2	3	1	Ser	TCT	2	3	4	Tyr	TAT	0	0	0	Cys	TGT	1	1	0
	TTC	0	0	0		TCC	2	2	0		TAC	0	1	1		TGC	0	0	0
Leu	TTA	1	1	1		TCA	0	1	1										
	TTG	2	0	0		TCG	2	0	0						Trp	TGG	1	1	1
Leu	CTT	4	4	5	Pro	CCT	2	1	0	His	CAT	3	3	0	Arg	CGT	1	0	0
	CTC	0	0	1		CCC	2	1	2		CAC	1	1	3		CGC	0	0	0
	CTA	1	1	0		CCA	0	0	0	Gln	CAA	0	0	3		GGA	1	1	0
	CTG	4	4	6		CCG	0	0	0		CAG	4	5	4		CGG	0	1	0
Ile	ATT	2	2	0	Thr	ACT	1	0	0	Asn	AAT	3	1	3	Ser	AGT	2	3	4
	ATC	0	1	1		ACC	0	0	3		AAC	2	2	1		AGC	1	2	1
	ATA	2	2	1		ACA	1	0	0	Lys	AAA	10	8	6	Arg	AGA	3	5	3
Met	ATG	6	7	8		ACG	0	0	0		AAG	4	6	6		AGG	2	0	3
Val	GTT	4	4	5	Ala	GCT	6	5	5	Asp	GAT	4	5	5	Gly	GGT	1	1	0
	GTC	2	2	3		GCC	1	2	2		GAC	3	3	1		GGC	0	1	4
	GTA	2	2	2		GCA	3	3	3	Glu	GAA	4	5	3		GGA	3	2	1
	GTG	3	4	3		GCG	0	0	0		GAG	3	1	3		GGG	1	1	2

Table 5
DIVERGENCE OF REPLACEMENT AND SILENT SITES BETWEEN BOVINE, HUMAN, AND RAT PTH GENES

Region of prePTH[a]	Bovine/human		Bovine/rat % Divergence[b]		Human/rat	
	Replacement	Silent	Replacement	Silent	Replacement	Silent
−31 to 84	7.4	16.8	14.1	33.2	14.8	27.6
−31 to −2	9.5	26.5	18.1	42.3	15.9	32.6
−1 to 29	4.5	28.8	9.1	29.9	10.6	29.2
30 to 59	8.7	12.6	18.3	39.6	19.1	31.3
60 to 84	7.1	10.1	12.9	24.7	14.7	18.8

[a] The regions of PTH were divided into 30 amino acid segments except for 25 amino acids in the last region. This divides the PTH molecule approximately into the prePro region, the biologically active region, and the N-terminal half and the C-terminal half of the biologically inactive C-terminal portions.

[b] Divergence was calculated as described by Perler et al.[57] except that no correction for multiple changes at a site was made.

third ATG codon of the rat sequence as the initiator codon is based on indirect evidence, primarily by comparison with the bovine and human cDNAs. Regardless, the presence of termination codons in phase with the earlier ATG prohibits the synthesis of a long protein initiated at these codons, as is the case in other genes with premature ATG codons.[54] A consensus for the sequence around the initiator codon of eukaryotic genes is CGACCATG.[54] The most stringent requirement for optimal initiation of synthesis is for a purine in the −3 position. Since none of the premature ATG codons in the rat and human has a purine in the −3 position, they are likely to be weak initiators. In contrast, the probable initiator ATG codon has an A at the −3 position in each sequence.

The codon usage for the three mammalian cDNAs is shown in Table 4. As has been observed for most eukaryotic genes, the usage of codons is decidedly uneven.[56] The low frequency of CG dinucleotides mentioned above reduces the codons containing CG. The four CGX codons of arginine, for example, are used 4 times in the three sequences while the two AGPu codons of arginine are used 16 times. The three PTH mRNAs have similar patterns of usage. Exceptions are the codons for His and Glu in which the usage for the rat gene differs from the other two. The reason for the difference in the usage of codons is not known.

A comparison of the amino acid sequences of PTH from several species revealed that three relatively conserved regions could be observed.[25] These included amino acids (−6) through 15, amino acids 23 to 38, and amino acids 49 to 84 (Figure 2). The first two regions would be expected to be conserved since they comprise the biologically active region of PTH. The addition or loss of a single amino acid at the amino terminus greatly reduces biological activity, and the region 23 to 30 is probably involved in binding of PTH to the receptor. In contrast, the C-terminal region, 49 to 84, is not biologically active, and the conservation in this region is somewhat surprising. An analysis of the silent changes that occur between the nucleotide sequences are suggestive that the conservation in the C-terminal region may be related to pretranslational events. Analysis as described by Perler et al.[57] of replacement changes that result in changes in amino acids and silent changes that do not alter the encoded amino acid is summarized in Table 5. The values are not corrected for multiple changes at a single site as described by Perler et al. because the correction assumes that transition and transversions occur at the same frequency. This is clearly not the case for the PTH genes. For example, for the 30 silent and replacement changes in the bovine and human cDNAs for which a change to any of the other three nucleotides is possible,

only 11 were transversions. Two thirds, or 20, would have been expected if the changes were random. The percentage changes in the table, then, are less than the actual mutation rate, but since the human, bovine, and rat species presumably diverged at about the same time, comparisons between the sequences are valid. About 7% of the possible replacement sites are changed between human and bovine PTH cDNA, while about 14% of the potential replacement sites were changed between rat and either the bovine and human sequences. The changes in silent sites follow a similar pattern with about 17% of sites changed for bovine/human and 27 and 32% for rat compared to human and bovine, respectively. Since the evolutionary distance between these sequences is about the same,[58] the increased rate of changes at silent sites, as well as replacement sites in the rat, suggest that the forces responsible for determining the rate of change act at the level of the gene or RNA as well as at the protein level.

A similar argument can be made to explain the conservation of protein sequence in the carboxyl terminal half of PTH. As shown in Table 5, the rate of replacement changes is reduced considerably as expected in the region, −1 to 29, which contains the biologically active region of PTH. Silent changes in this region are generally near or above the average for the entire coding region. In contrast, in the conserved C-terminal region (60 to 84), replacement changes are above average, but, surprisingly, silent mutations are also lower than the average for the entire cDNA. This is also true for the C-terminal region 30 to 59 in the bovine/human comparison. This suggests that there are constraints in changes on the RNA sequence in this region, and that the unexpectedly low number of replacement sites in this region may result from constraints on both nucleic acid and protein structure.

The rate of silent changes for the entire coding region is less than expected. Silent changes have been estimated to be about 1%/million years.[57] If the mammalian radiation occurred about 80 million years ago,[58] a change of about 80% corrected for multiple events would be expected. The human-bovine difference of 17% is clearly much less than this even though it is uncorrected. On the other hand, changes corrected by the Perler et al.[57] method, are 62 and 84% for the rat compared to the human and bovine, respectively, which is closer to expected rate of divergence. The unexpectedly low divergence at silent sites also suggests constraints on changes in the RNA. Conformation, processing, or stability of the RNA could potentially require conservation of sequence.

E. 3′ Untranslated Region

As noted above, the 3′ untranslated region is the most variable region of the cDNAs requiring significant gaps to maximize homology. The termination codon in the human and bovine is TGA and is followed closely by a second in-phase termination codon. In the rat TAA is the termination codon, which is rather rare in mammalian mRNAs[59] and no following termination codon is present. One region that is well conserved in the three sequences is the AATAAA sequence that is thought to be a signal for polyadenylation in eukaryotic mRNAs. In the bovine sequence, only a single AATAAA has been detected in the 3′ noncoding region, whereas in the human and rat sequences two potential polyadenylation sites are found. The functional polyadenylation signal in the human cDNAs has 11/12 and 9/12 matches with the bovine and rat sequences, respectively. The second AATAAA region in the human sequence is about 60 bases upstream from the first and has been suggested to have resulted from a gene duplication;[23] however, other than the AATAAA regions, there is little homology surrounding the two sites. Sequence analogous to the human upstream AATAAA is missing in both the bovine and rat sequences. No cDNAs were detected in which the upstream AATAAA was utilized as a polyadenylation signal; however, only a small number were analyzed, and the probability that these sites function as a polyadenylation signal cannot be ruled out. The rat sequence also has a second AATAAA site about 115 nucleotides earlier than the functional one. A single rat PTH mRNA was detected by Northern

blot analysis,[25] suggesting only one polyadenylation site is used, and the size of the mRNA was consistent with the second AATAAA being the site. There is no direct evidence for the location of the 3′ end by analysis of the rat PTH mRNA or cDNA.

Other than the AATAAA sequence itself, little homology is observed at the 3′ end of the cDNA between the rat sequence and the other sequences. In contrast, the 3′ terminus of the human and bovine cDNAs in the regions around the polyadenylation signal and the polyadenylation site are identical in 23 out of 24 positions, with the exception that a five nucleotide gap must be introduced in the human sequence between the signal and site. Even though the sequences in the region of the polyadenylation sites are identical in bovine and human, the human site is offset about five nucleotides, thus maintaining the same distance between the signal and site in both sequences. This indicates that the actual polyadenylation site is determined more by the distance from the polyadenylation signal than the actual sequence at the site. The polyadenylation site for the rat sequence as shown in Figure 2 was chosen so that a similar distance between the signal and site is maintained and is four or five nucleotides prior to that proposed previously.[25]

V. PTH GENES

The genes for human,[24] bovine,[22] and rat[25] PTH have each been cloned and characterized from genomic libraries in lambda phage. The human gene was isolated from a total human fetal DNA library prepared in λ phage Charon 4A. The library was screened initially by filter hybridization with human cloned cDNAs as a probe and later by the recombination selection method. The structure of the human gene was determined by the analysis of two overlapping clones. For the bovine gene, Southern analysis of the total bovine DNA showed that the PTH gene was present on an 8000 bp EcoR1 fragment. To clone the gene, bovine liver DNA was digested with EcoR1 and fragments in the range of 5,000 to 10,000 bp were isolated by sucrose gradient centrifugation. A partial library was then constructed by ligating the EcoR1 fragments to λ phage Charon 31 arms, which had been isolated after digestion with EcoR1. Several independent clones were isolated by plaque filter hybridization using cloned bovine PTH cDNA as probes. Two of these clones were characterized in detail. Both were identical and contained the entire gene sequence. The rat gene was isolated from a λ phage Charon 4A rat liver DNA library produced by partial EcoR1 digestion of the rat DNA. Two independent positive plaques out of the 200,000 plaques screened were obtained. The insert of each of the two phage contained the entire rat PTH gene.

A. Structure of the Genes

The overall structure of the bovine PTH gene is shown at the top in Figure 3. All three of the genes contain two introns. The exact location of the bovine and human gene introns were determined by comparing the sequence of the gene to the previously determined cDNA structure. The location of intron A of the rat was determined by comparing the gene sequence with the sequence of cDNA to the 5′ end of the mRNA. The cDNA was synthesized with reverse transcriptase using a synthetic pentadecamer as primer. Intron B in the rat gene was determined indirectly by homology of sequence with the human and bovine cDNA sequences.

The location of the introns are identical in each case as has been found with most other genes.[61] Intron A splits the 5′ untranslated sequence five nucleotides before the initiator methionine codon (Figure 2). Intron B splits the fourth codon of the region that codes for the pro sequence of preProPTH. The three exons that result, thus, are roughly divided into three functional domains. Exon 1, 95 to 121 nucleotides, contains the 5′ untranslated region. Exon 2, has 91 nucleotides and codes for the pre sequence, or signal function, and exon 3, 375 to 486 nucleotides, codes for PTH as well as the 3′ untranslated region. The structure of the PTH gene is thus consistent with the proposal that exons represent functional domains of the mRNA.[62]

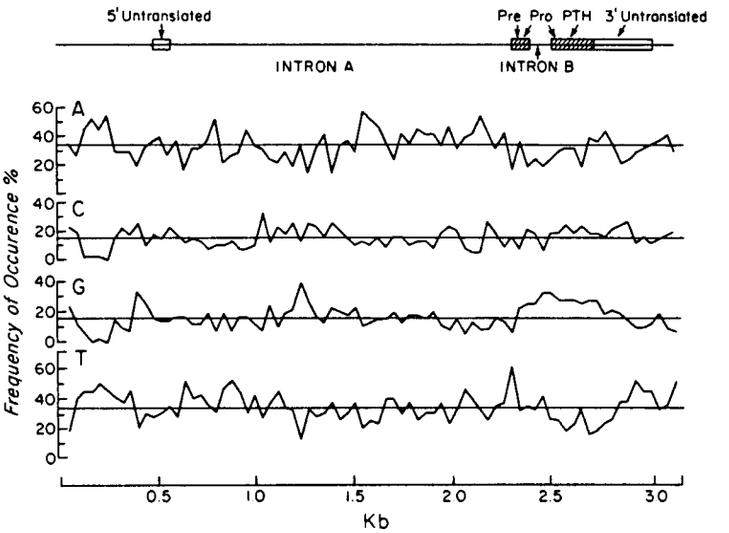

FIGURE 3. Schematic diagram of the bovine PTH gene structure and the frequency of occurrence of the four deoxynucleotides. Exons are indicated by the rectangles and the shaded area indicates regions of the gene that code for preProPTH. The plot of the frequency of occurrence of each nucleotide was generated by plotting the percent of each nucleotide in blocks of 40 nucleotides. The horizontal line indicates the average percentage of the nucleotide in the entire gene.

Although the introns are in the same location, the size of the large intron A in the human is about twice as large as those in the rat and bovine. Sequence for the entire intron is not available for the human sequence so that the nature of the extra sequence is not known. It is interesting that the human gene is considerably longer in both intron A and the 3′ untranslated region of the cDNA than the other two sequences. Knowledge of the structures of other PTH genes from other species will be necessary in order to determine whether extra sequence was inserted or is less susceptible to deletion in the human gene.

In each of the species, only a single PTH gene appears to be present. Extensive Southern blot analysis of bovine DNA with cloned PTH cDNA as probe produced single hybridizing bands for restriction enzymes that do not cut within the probe sequence.[22] The restriction map determined from the Southern analysis of bovine DNA was consistent with that of the cloned gene. With the exception of a single nucleotide in the 3′ untranslated region, the sequence of the cloned cDNA was identical to the sequence of the exons in the gene. Less extensive Southern blot analysis of the human[24] and rat[25] genes also were consistent with a single gene per haploid genome. Furthermore, in the human studies, probes from the 5′ and 3′ ends of the cDNA both hybridized to the same sized fragment, and the strength of the signal from the genomic DNA was about the same as one gene-equivalent of the gene cloned in λ phage. No evidence has been obtained for PTH pseudogenes or other closely related genes. Thus, the PTH gene, in contrast to many other genes, is a single gene with no other closely related functional or nonfunctional relatives.

The complete nucleotide sequence of the bovine gene has been determined and a base composition profile is shown in Figure 3. Overall, the gene is very AT-rich, containing 68% of these nucleotides. The region around exons 2 and 3 and intron B are relatively G rich and contain the lowest percentage of A and T. Two other striking G rich regions occur just before exon 1 and in the middle of intron A. In general, the frequencies of G and T are reciprocal. The significance of the different base compositions in the various regions of the gene is not clear.

FIGURE 4. Comparisons of nucleotide sequences of regions from the bovine, human, and rat PTH genes. Gaps indicated by the dahses were introduced to maximize homology. Numbering is arbitrary because of the gaps. Nucleotides in one sequence that are different from those in both the other two sequences are underlined. In the 5′ flanking region, the potential Z-DNA forming region containing repeating GT sequence, and the two TATA sequences are overlined. In intron A only regions sequenced for all three species are shown. Intron A contains 1714 bp, about 1600 bp and about 3500 bp in the bovine, rat, and human genes, respectively. The exact positions of the intron/exon junctions are ambiguous from the comparison of the gene and cDNA sequences and are placed to conform with the GT/AG rule.FIGURE 61. In the 3′ flanking region, the polyadenylation signal is overlined and polyadenylation sites are indicated by a dot. A potential hairpin loop in the transcript is indicated by the dashes (stems) and dots (loop). This region is followed by several Ts, which is characteristic of transcription terminators in prokaryotes.

B. Initiation Site for RNA Transcription

As noted above in the discussion on the cDNA, the 5′ termini of bovine PTH mRNA are heterogeneous. The large mRNAs contain a TATA sequence in the appropriate location to direct the synthesis of the smaller mRNAs. It was postulated that a second TATA would be found in the gene sequence 5′ of the first one.[21] As shown in Figure 4, in both the human and bovine gene sequences, a second TATA sequence is present in the 5′ flanking region about 25 base pairs from the first one in the appropriate position to direct the synthesis of the larger mRNAs. The heterogeneity of the 5′ end of the bovine PTH mRNA, originally

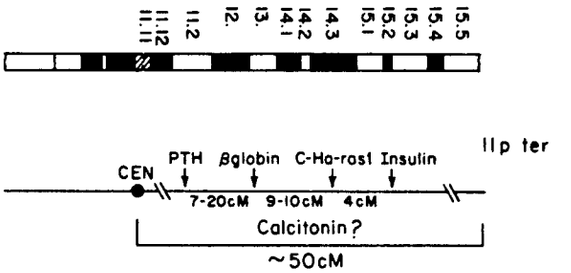

FIGURE 5. Chromosomal location of the human PTH gene. A schematic diagram of the short arm of chromosome 11 is shown at the top and the estimated distances in centimorgens (cM) between the gene loci is shown below. 1 cM is equal to about 10^6 bp. (The figure is adapted from Antonarakis et al.[76])

detected by reverse transcription of the mRNA,[21] was confirmed by S1 nuclease mapping.[22] The initiation sites for human PTH mRNA have not been determined directly, but were proposed[25] on the basis of analogy with the bovine sequence and the consensus TATA sequences. The presence of multiple functional TATA sequences has been reported for several other eukaryotic genes.[63-65] The rat mRNA appears to be relatively homogeneous at the 5′ terminus on the basis of both primed reverse transcriptions near the 5′ end of the mRNA and S1 nuclease mapping.[25] The single initiation site for the rat mRNA can be explained by the changes in the rat sequence which alter the second downstream TATA sequence. The sequence, TATATATAAAA, in the human and bovine genes, is changed to TGCATATGAAA in the rat gene (Figure 5), which is no longer a consensus TATA sequence. While this change seems the most likely explanation for the difference in length at the 5′ termini between the mRNAs, there are other changes that also occur in this region of the gene and might play a role.[25] The loss of the second TATA sequence makes it unlikely that the presence of two of these sequences in the human and bovine genes rather than one is essential for proper transcription of these genes.

The smaller bovine PTH mRNAs are also heterogeneous with initiation occurring over a range of about eight nucleotides at the 5′ terminus. The second TATA sequence in the bovine sequence is unusual since the sequence TA is repeated five times, and thus the TATA-like sequence is spread over 12 base pairs. This may result in a less rigorous delineation of the appropriate start site.

The conclusion that the 5′ end of bovine mRNA is heterogeneous has not been conclusively proven. Both the S1 nuclease mapping and the primed reverse transcriptase techniques require that the mRNA is intact and not degraded. Since in the studies described above, it was not demonstrated that all the mRNA had a 5′ methylguanosine cap and, thus, was intact, the possibility that heterogeneity was introduced during isolation of the mRNA cannot be excluded. However, the additional indirect evidence provided by the presence of two TATA sequences considerably strengthens the theory that two regions are utilized for initiation of transcripts.

C. 5′ Flanking Region

The homology among the three genes for about 300 bp in the 5′ flanking region is illustrated in Figure 4. Homology in this region, particularly for the first 200 bp upstream of the RNA initiation site, is similar to that in the 5′ untranslated region of the mRNAs (Table 3). There are few stretches of sequence in the 5′ flanking region that are completely conserved in all three sequences except for the TATA sequences. The C-rich sequence, $GCACCGCCC_{143}$, about 75 bp to the 5′ side of the upstream TATA sequence is present in all three sequences,

and an AT-rich region of about 25 bp immediately prior to this C-rich region is strongly conserved. The sequence, $CAGAGAA_{194}$, about 25 bp to the 5′ side of the TATA sequence, is also present in all three sequences. These sequences do not closely resemble upstream elements conserved in other genes, although a C-rich sequence at −100 is important for maximal transcription of the Herpes simplex virus thymidine kinase gene.[66] No CAAT sequence such as that found in globin and other genes is present 5′ of the TATA sequences. A short stretch of repeating GTs at about position 150 (Figure 4) is found in the human gene and is partially conserved in bovine, but is not present in rat. Since this type of sequence can form Z-DNA[67] and Z-DNA has been observed in regulatory regions of genes,[68] there is a possibility that this region might be involved in the regulation of the gene. In the bovine gene, an extraordinary stretch of almost 150 nucleotides, located from 250 to 400 nucleotides before the transcript initiator, consists primarily of alternating AT (not shown). A similar region is not present in the rat gene, suggesting it is not critical for the function of the gene. On the other hand, AT-rich regions far upstream from the promoter have been reported to be required for the transcription of other genes.[69,70] AT-rich regions in the 5′ flanking regions of *Drosophila* heat shock genes have been proposed to function as attachment sites to the nuclear scaffold.[71] Such regions have also been proposed as the boundaries between gene domains.[72] These last two hypotheses suggest that the AT-rich region may mark the 5′ end of the functional PTH gene. The significance of the 5′ flanking sequence in transcription will eventually require an analysis of the expression of cloned natural genes and mutated genes after transfection into cells in culture.

D. Introns

Both introns follow the GT-AG rule for the sequence at the 5′ and 3′ ends of the intron.[61] In addition, both have pyrimidines near the 3′ end of the introns as is usually the case. Splicing of pre-mRNA probably involves the formation of a lariat-like intermediate, which for globin and adenovirus RNA precursors involves an interaction of the 5′ end with sequence about 30 nucleotides from the 3′ end of the intron.[73,74] There is no obvious homology in the PTH genes with these acceptor regions in the globin genes near the 3′ end of the introns.

A complete sequence for the large intron A is available for the bovine gene. The intron is quite rich in As and Ts containing 70% of these nucleotides, a property shared with the 5′ flanking regions. A G-rich sequence is present in about the middle of the intron A (Figure 2). About 170 bp from the 3′ end of the bovine gene intron A, a tandem triplication of 28 base pairs forms an AT-rich region that is similar to the long AT-rich region in the 5′ flanking region (not shown). It is interesting to speculate that these two regions might be remnants of direct repeats formed in an ancestral gene when a mobile element carrying the 5′ flanking region and exon 1 was inserted near the other PTH exons.

About 75% homology over 200 bp of intron A is observed for the human and bovine genes if gaps are considered a single mismatch. This degree of homology is about the same as that in the other untranslated regions of the genes. The rat intron A sequence is less homologous to the other two, 55 to 57% again similar to that of the other untranslated regions of the genes. The sequence of introns are generally only conserved very near to the exon/intron junction,[61] and 80 bp has been shown to be the minimum size for a functional intron in a globin gene.[75] The larger stretches of homology for the PTH genes suggest that there may be some constraints on the base changes some distance from the intron/exon border.

Intron B containing 106 to 121 bp is much smaller and more homogeneous in size among the genes than Intron A. The sequence of intron B is well conserved with homology of 74 and 68% of bovine/human and human/rat, respectively, but is relatively poorly conserved between rat and bovine genes with a homology of 49%. Intron B in the bovine gene contains nearly 30% Gs, which is more than any of the other units of the gene. It is interesting that

exon 2 and the coding region of exon 3, which flank intron B, also have relatively high G contents of 23 and 26% compared to other regions of the gene.

E. 3′ Flanking Region

In the 3′ flanking region, again there is also considerable homology between the bovine and human sequences (Figure 3, Table 4), in spite of the observations that only about 20 bp are required for appropriate polyadenylation of globin[76] and growth hormone[77] mRNAs. A sequence that resembles a prokaryotic transcription stop signal is present about 100 bp following the polyadenylation sequence in the human and bovine sequences. A small inverted repeat region, that could form a hairpin loop in the transcript, is followed by a stretch of 7 Ts. A difference in the stem in the human compared to bovine is matched by a second change in the human that maintains the base pairing in the stem. There is no direct evidence that this region serves as a transcriptional stop signal in the PTH genes. A similar sequence is not present in the approximately 110 bp of 3′ flanking sequence reported for the rat sequence. The rat in fact has little homology with either of the other two sequences beyond the polyadenylation signal (Figure 4, Table 3). This is surprising in view of the homology retained between the rat PTH gene and the other genes in the 5′ flanking and intron regions. Perhaps the polyadenylation signal for the rat sequence is derived from a different region of the gene, which was moved into its present position by a deletion of sequence or translocation. Large gaps must be introduced into the bovine and rat sequences just prior to the polyadenylation signal supporting the idea that this may be a relatively unstable region of the gene.

Overall, the PTH genes are typical eukaryotic genes that contain the consensus sequences for initiation of RNA synthesis, RNA splicing, and polyadenylation. Perhaps the most striking characteristic of the DNA in the region of the genes is its stability. In contrast to many genes, the PTH genes appear to be represented only once in the haploid genome. There is no evidence for related genes produced by gene duplication nor for pseudogenes produced by any mechanism. In addition, regions that diverge rapidly in other genes are relatively stable in the PTH genes, particularly between the human and bovine sequences and to a lesser extent with the rat sequence. Thus, considerable homology is observed between 5′ and 3′ flanking and untranslated regions, internal regions of introns, and potential sites for silent changes in the coding region. Since these regions that do not change the amino acid sequence have been estimated to diverge at a rate of $1\%/10^6$ years, relatively low homologies would be expected from these sequences that diverged about 60 to 80 $\times$ 10^6 years ago. Whether this conservation of sequence occurs because the genes happen to be present in a region of the chromosome that is usually stable or reflects some functional constraints inherent in the PTH gene, remains to be elucidated.

The rat sequence is considerably less homologous to the human and bovine sequences than these sequences are to each other. This observation is difficult to explain, since evolutionarily each of the sequences are about equidistant from another. Potentially, differences in the physiology or nutrition of calcium in the rat compared to the other two species might have resulted in increased acceptance of mutations in the rat PTH gene.

VI. CHROMOSOMAL LOCATION OF THE HUMAN PTH GENE

The location of the human PTH gene on chromosome 11 has been determined independently by two groups. The assignments were made by screening panels of human-mouse[78] or human-mouse and human-Chinese hamster cell[79] hybrids with a human cDNA clone or a cloned fragment of human genomic DNA. In both cases, complete concordance was observed with both lactate dehydrogenase-A, which has been assigned to chromosome 11, and with the presence of chromosome 11 determined by cytological techniques. The PTH

gene was further localized to the short arm of the chromosome 11 by analysis of human-mouse hybrids with various translocations.[78] Three hybrids contained the long arm and centromere, but not the short arm of chromosome 11, and in these cases PTH cDNA did not hybridize to the DNA from these hybrids. DNA of another hybrid cell line that had both chromosomes resulting from a translocation of the X chromosome, and chromosome 11 also did not hybridize to PTH cDNA.[79] This hybrid was reported to contain a deletion between 11p11 and 11p13, suggesting that the PTH gene was in this region.

Two polymorphisms have been detected near the PTH gene in the human population.[80,81] In both cases the polymorphism was detected by changes in the lengths of fragments generated by restriction enzymes. The restriction enzyme, Pst 1, generated fragments containing exon 3 of either 2.2 kb and 2.7 kb in different individuals. The larger fragment was generated by the loss of a Pst 1 site in the 3′ flanking region of the PTH gene. The frequency of the presence of the Pst 1 site that generated the 2.2 kb fragment ranged from 0.25 to 0.62 in several ethnic groups and was 0.42 in a population of northern Germans.[80,81] The restriction enzyme Taq 1 generated a constant fragment of 4.1 kb continuing exon 3 and either a 2.4 kb or 2.5 kb band containing exon 2.[81] The polymorphism was due to the absence or presence of a Taq 1 site in the small intron B. In the northern German population, the frequency of the presence of the Taq 1 site was 0.63.[81] Thus, both polymorphisms occur at a relatively high frequency. Furthermore, the resulting alleles segregate in Mendelian fashion, and the distributions of the alleles agree with the Hardy-Weinberg law. These polymorphisms thus should be useful for analysis of gene linkage as well as correlating the presence of the polymorphism with parathyroid disease that is based on an alteration of the PTH gene.

The short arm of chromosome 11 contains several other polymorphic genes including the β globin gene cluster, insulin, and the human oncogene C-Ha-ras-1.[82,83] The polymorphisms in these genes and PTH were used to determine whether the genes are genetically linked and their order on the chromosome. The order of the genes and their relative distances are shown in Figure 5. In addition to these genes, the gene for calcitonin has also been mapped to the short arm of chromosome 11, although its location relative to the other genes is not yet known.[84] Thus, the short arm contains genes for both of the polypeptide hormones that regulate calcium metabolism. Whether this is a mere coincidence or is somehow related to the evolution or regulation of these calcium regulating genes remains a matter of speculation.

VII. REGULATION OF PTH mRNA LEVELS

A. Parathyroid Slices Incubated In Vitro

Studies on the synthesis of PTH in slices of parathyroid glands or dispersed cell culture have generally indicated relatively small changes in the rate of synthesis of the hormone after acute changes of calcium, in contrast to rapid and large effects on secretion.[5] The levels of PTH mRNA, thus, would not be expected to change greatly in parathyroid tissue under similar conditions. This has been confirmed by a study of the concentration of PTH mRNA in slices of bovine parathyroids incubated in vitro for 5 to 7 hr with various concentrations of extracellular calcium.[85] The bovine PTH mRNA was quantitated by Northern blot analysis of total or poly(A) containing parathyroid RNA. No differences in the amount of total mRNA were observed between tissue incubated with high or low levels of calcium. Treatment of the tissue with concentrations of Actinomycin D, which inhibited total RNA synthesis by 90%, did not alter PTH mRNA levels over a 6-hr period, indicating that the PTH mRNA is quite stable under these conditions. The levels of poly(A) containing PTH mRNA were affected by extracellular calcium levels. Paradoxically, low concentrations of calcium, which stimulate PTH secretion, reduced the amount of poly(A) containing PTH mRNA measured either by translation or RNA blot hybridization. Poly(A) containing PTH mRNA was only 10% of total RNA from tissues treated with low calcium and 30% in tissues

treated with high calcium. Total nucleic acids labeled with ^{14}C-uridine and ^{3}H-labeled polyadenylic acid added exogenously during the isolation procedure were recovered equally well in both samples. The 3′ poly(A) region of mRNA may stabilize mRNAs,[86] and newly synthesized mRNA should have longer poly(A) extensions. Therefore, the observation that the fraction of poly(A) containing PTH mRNA is reduced when the gland is being stimulated is the opposite of the expected and remains an enigma.

Since translation of total poly(A) RNA and that of parathyroid secretory protein and actin followed the same pattern as that of PTH, the effect on poly(A) content is clearly not specific for PTH mRNA alone. In either high or low calcium, most of the PTH mRNA does not bind to oligo(dT) cellulose, a fact noted previously,[47] yet the PTH mRNA appears to be very stable. Factors other than the poly(A) extension are probably responsible for the stability of the mRNA.

There are two major drawbacks to the parathyroid slice system for analyzing PTH biosynthesis. The slices are relatively thick so that the metabolic activity of cells in the interior of the slices is likely to be different from that of cells on the exterior. Nevertheless, this system has been very valuable in elucidating the biosynthetic pathway of PTH, and there is no direct evidence that artifacts have been introduced by the thickness of the slice. The second disadvantage is that the slices have been used only in acute experiments of shorter than 5 to 7 hr. Since chronic stimulation in vivo is known to result in enlargement of the parathyroid gland and increased production of PTH, long-term culture systems are necessary to provide in vitro models of chronic stimulation.

B. Dispersed Cell Culture

The development of a dispersed cell culture system in which regulation of PTH synthesis can be studied over several days represents a significant advance in PTH biosynthetic studies.[87] In dispersed bovine parathyroid cells, the levels of PTH mRNA were measured by RNA solution hybridization or RNA dot blot hybridization with a cloned bovine PTH cDNA probe. In these cells, the level of PTH mRNA was reduced to a plateau of about 30% by 72 to 96 hr in cultures in 2.5 m*M* Ca^{++} compared to those in 0.5 m*M* Ca^{++} (Figure 6). If the high calcium medium was replaced by low calcium medium at 36 hr, the decrease was reversed. Neither total RNA nor ^{3}H-uridine labeled RNA was significantly different in the high and low calcium samples. These results strongly suggest that chronic suppression of the parathyroid tissue leads to a specific decrease in the levels of PTH mRNA. Additional experiments are necessary to determine whether the decrease in PTH mRNA is due to decreased synthesis or increased degradation. It is interesting that lower than normal concentration of Ca^{++} (0.5 m*M*) did not affect PTH mRNA levels when compared to normal concentration of calcium (1.25 m*M*). This is unexpected, since chronic stimulation of glands in secondary hyperparathyroidism leads to excess PTH production. It is possible that the stimulatory effect is on general parathyroid cellular growth and metabolism rather than a specific effect on PTH synthesis. This cell culture system should be a useful model system in further studies on PTH biosynthesis. One note of caution is the observation that secretion of PTH is suppressed to only 50% of control, while in parathyroid slices incubated in vitro and in vivo, suppression to about 20% is observed.[4,88] It is possible that cell-cell interactions in the gland are important for the proper regulation of cellular activity, and this characteristic would be lost in the dispersed cell cultures.

VIII. MEDICAL APPLICATIONS OF PTH MOLECULAR BIOLOGY

There are a number of diseases that involve abnormal production of PTH. Whether these diseases are related to changes in the structure of the PTH gene or its chromosomal environment is not known. The demonstration of polymorphisms around the PTH gene that

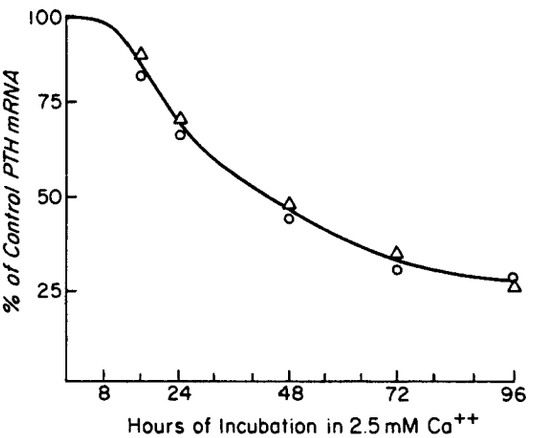

FIGURE 6. Decrease of PTH mRNA in primary cultures of bovine parathyroid cells cultured in high (2.5 m*M*) calcium. The mRNA was assayed by either solution hybridization (△) or RNA dot blot hybridization (○). Control incubations were at 1.25 m*M* calcium. Incubations at low calcium (0.5 m*M*) were not significantly different from the controls (From Russell, J., Letteri, D., and Sherwood, L. M., *J. Clin. Invest.*, 72, 1851, 1981. With permission.)

occur with high frequency should make it possible to correlate particular alleles with the parathyroid disease.[80,81] If such a correlation exists, then not only is significant information about the basic molecular defect obtained, but this information may be used to screen for or diagnose the disease.

In another medical application, hybridization with PTH cDNA has been used to try to detect PTH mRNA in tumors associated with hypercalcemia.[89] Some nonparathyroid solid tumors are associated with hypercalcemia hypophosphatemia and increased urinary cAMP, all of which are characteristic of excess parathyroid hormone production.[90] Attempts to detect elevated PTH levels by immunoassay led to contradictory conclusions about the production of PTH by these tumors.[89] Since these differing results may have occurred because of technical differences with the radioimmunoassay, an assay for PTH mRNA was used as an independent method of analysis. Only mRNA preparations that directed the synthesis of proteins in a cell-free system were analyzed to control for degradation of the mRNA during preparation. In a variety of tumors, human and animal, no PTH mRNA was detected by Northern blot analysis using both bovine and human cDNAs. Some of these tumors were ectopic tumors with clinical symptoms suggesting the production of a PTH-like molecule. These results suggest that tumors that produce PTH are relatively rare and that the hypercalcemia usually results from other factors produced by the tumors.

PTH may be useful in treating a variety of bone diseases and has been used experimentally to treat osteoporosis.[91] The therapeutic usefulness of PTH is limited by a short supply of the hormone, particularly human PTH. The synthesis of human PTH in bacteria, which may remedy this problem, has been reported recently.[92] A Sau3A restriction fragment of human PTH cDNA was used that contained the coding sequence for PTH. An initiator methonine codon was introduced by ligation of a synthetic octomer at the 5′ end, and the construction was cloned into the Cla I site of pBR322. Small deletions from the nearby Hind III site in pBR322 followed by ligation of a decamer containing the bacterial ribosomal binding site produced a set of constructions with 6 to 10 bp between the initiator ATG codon and the ribosomal binding site. These constructions were then subcloned downstream from the strong

bacterial promoters, trp, tac, or lac. Production of PTH was determined by radioimmunoassay. Maximal synthesis of PTH occurred in constructions containing 9 bp rather than 4 or 5 bp between the ribosomal binding site and the ATG codon. Up to 200 μg PTH per liter of culture medium was obtained. The slope of the competitive binding curves of the bacterial extracts and human PTH (1 to 84) to the PTH antisera were essentially identical. The biological activity of the recombinant PTH was assayed by the renal adenyl cyclase assay, and the amount of biologically active PTH present was about the same as the amount determined by radioimmunoassay. Since f-met at the N-terminus of the recombinant PTH should decrease biological activity, it is possible that f-met either does not diminish activity or more likely that the f-met is removed from the PTH molecule by bacterial enzymes. The accomplishment of obtaining human PTH from bacteria gives promise of the availability of large quantities of the hormone for therapeutic purposes.

IX. SUMMARY

The entire biosynthetic pathway of PTH has been elucidated from the determination of the chromosomal location to the eventual secretion of the hormone from the cell. The human gene is present on the short arm of chromosome 11, and restriction site polymorphisms near the gene have been detected. The PTH genes and cDNAs have been isolated and characterized in the bovine, human, and rat species. The gene contains two introns, which are in the same position in each species, and dissect the gene into 3 exons that code, respectively, for the 5′ untranslated region, the signal peptide, and PTH plus the 3′ untranslated region. The mRNAs are about twice as long as necessary to code for preProPTH and contain a 7-methylquanosine cap at the 5′ terminus and polyadenylic acid at the 3′ terminus. The 5′ termini of the bovine and human mRNAs are heterogeneous at the 5′ terminus, the basis of which is two TATA sequences in the 5′ flanking regions of the gene. In contrast, the rat gene contains a single TATA sequence and the mRNA has a single 5′ terminus. The initial translational product of the mRNA is preProPTH, and the pre-peptide of 25 amino acids is equivalent to signal peptides of other secreted and membrane proteins.

The genes of the three species are very homologous in the region that codes for preProPTH. Substantial homology is also retained in the gene flanking regions, introns, and mRNA untranslated regions. Silent sites are also conserved more than would be expected, particularly between the human and bovine sequences. The bovine and human sequences are more closely related than the rat is to either the human or bovine.

These studies of the basic molecular biology of PTH will provide the framework for future analysis of significant biological and medical questions. In vitro mutagenesis techniques should soon provide information about the elements of the gene involved in regulating transcription and about functional elements of the signal peptide. Eventually, signals involved in directing the ProPTH molecule to secretory granules as well as the biologically active regions of PTH, itself, will be examined by these methods. The molecular biological studies, combined with the development of dispersed cell cultures, provide the opportunity to study the effects of chronic changes in calcium on gene transcription and mRNA metabolism. The restriction site polymorphisms associated with the human PTH gene will allow a search for correlations between PTH gene structure and parathyroid disease. Finally, the production of active PTH in bacteria may provide large amounts of PTH for testing its possible therapeutic efficicacy in a variety of diseases that affect calcium and bone metabolism.

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