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Alternative processing of bovine growth hormone mRNA: Nonsplicing of the final intron predicts a high molecular weight variant of bovine growth hormone

(intron D/alternative reading frame/growth hormone-related polypeptide)

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ABSTRACT **We have detected a variant species of bovine growth hormone mRNA in bovine pituitary tissue and in a stably transfected bovine growth hormone-producing cell line. Analysis of this variant mRNA indicated that the last intervening sequence (intron D) had not been removed by splicing. Inspection of the sequence of intron D reveals an open reading frame through the entire intron, with a termination codon encountered 50 nucleotides into the fifth exon, which is shifted from the normal reading frame in this variant mRNA. If translated, this variant mRNA would encode a growth hormone-related polypeptide having 125 amino-terminal amino acids identical to wild-type growth hormone, followed by 108 carboxyl-terminal amino acids encoded by the 274 bases of intron D along with the first 50 nucleotides of exon 5. This variant polypeptide would be 42 amino acids longer than wild-type bovine growth hormone or approximately 5000 greater in molecular weight. The intron D-containing variant of bovine growth hormone mRNA was demonstrated to exist on polysomes, suggesting that this mRNA species is translated into a polypeptide. Cytosolic mRNA species containing any of the other three introns of the bovine growth hormone gene were not detectable.**

Growth hormone is a M_r 22,000 polypeptide hormone secreted by somatotrophs of the anterior pituitary, resulting in long-term stimulation of growth as well as a number of short-term metabolic effects (1). The gene coding for bovine growth hormone is approximately 1800 base pairs in length, composed of five exons interrupted by four introns (2). The mature mRNA is 814 nucleotides long (excluding polyadenylylation) containing 58 and 105 nucleotides of 5′ and 3′ untranslated regions, respectively. Bovine growth hormone is synthesized as a 217 amino acid precursor that is subsequently processed proteolytically to the mature polypeptide 191 amino acids long (2).

Alternative processing of mRNA precursors is an important mechanism in generating diversity of gene expression (3–5). Multiple splicing patterns of a mRNA precursor can lead to different protein products from a single gene, including genes coding for hormonal polypeptides. We have detected a novel species of bovine growth hormone mRNA from which the final intron (intron D) is not removed. Preliminary evidence suggests this variant mRNA is translated into a growth hormone-related polypeptide that would be approximately 5000 greater in molecular weight than wild-type growth hormone. This variant mRNA has been detected in bovine pituitary tissue as well as in a stably transfected bovine growth hormone-producing cell line (CHO 14-10-4).

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MATERIALS AND METHODS

CHO 14-10-4 Cell Line. The Chinese hamster ovary (CHO) cell line, CHO 14-10-4, utilized in these studies was generously provided by Leonard Post. These cells were derived from the DBX-11 cell line of dihydrofolate reductase-negative CHO cells (6) and have been stably transfected with an expression plasmid containing the bovine growth hormone gene. This expression plasmid, pSV2Cdhfr (Fig. 1), contains the *Bam*HI/*Eco*RI fragment of the bovine growth hormone genomic clone (2) in the plasmid pSV2dhfr (7) situated downstream from a 760-base-pair *Sau*3A fragment containing the human cytomegalovirus immediate early promoter (8). Clones of cell lines were adapted stepwise to growth in 3 μM methotrexate in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and nonessential amino acids.

Isolation of RNA. The CHO 14-10-4 cells were harvested in phosphate-buffered saline (PBS) containing 1 mM EDTA. Nuclear and cytoplasmic cell fractions were isolated (9), and RNA was extracted by the guanidinium isothiocyanate/cesium chloride centrifugation method (9). Polyadenylylated RNA was prepared by chromatography on oligo(dT)-cellulose as described (9).

For preparation of polysomal RNA, the cells were treated with emetine at 100 μg/ml for 10 min at 37°C prior to harvesting to prevent ribosome run-off during the isolation procedure. Polysomes were sedimented through sucrose density gradients and fractionated with an Isco gradient fractionator equipped with a flow cell. Details of this procedure are described elsewhere (10). Polysomal fractions from bovine pituitary tissue were prepared from tissue slices. The anterior pituitary was dissected from the gland, and slices 1 mM thick were prepared by using a Stadie–Riggs tissue slicer. The slices were incubated at 37°C for 60 min with constant agitation in Petri dishes covered by a thin layer of DMEM supplemented with 0.35% glucose. After incubation, 100 μg of emetine per ml was added, and the slices were incubated for a further 10 min. Extracts of the pituitary tissue slices were prepared essentially the same as from the CHO 14-10-4 cells except that the tissue slices were homogenized at low speed in a Potter–Elvehjem homogenizer.

Analysis of RNA. Polyadenylylated RNA was separated by electrophoresis in formaldehyde gels (11), transferred to Zeta Probe (Bio-Rad) by standard blotting procedures, and hybridized as described (12). Nuclease S1 protection experiments were performed as described (12). Hybridization probes were prepared from phage M13 templates as described elsewhere (13).

Abbreviation: CHO, Chinese hamster ovary.

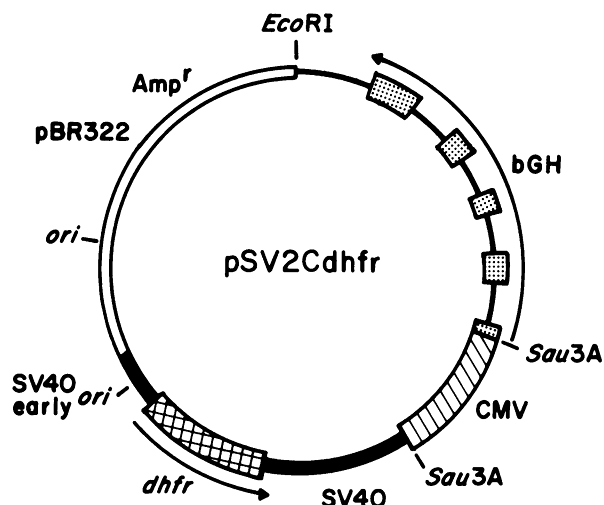


FIG. 1. Structure of the expression plasmid pSV2Cdhfr used to create the CHO 14-10-4 cell line. Relevant portions of the plasmid are as indicated. CMV, immediate early promoter of human cytomegalovirus; bGH, bovine growth hormone structural region; *dhfr*, dihydrofolate reductase gene; SV40, simian virus 40; *ori*, origin of translation; Amp^r, gene encoding ampicillin resistance.

RESULTS

The availability of a stable cell line producing significant levels of bovine growth hormone mRNA provided a potential system to investigate the processing of bovine growth hormone mRNA. The stably transfected bovine growth hormone-producing cell line, CHO 14-10-4, utilized in this investigation was obtained by stable incorporation of an expression plasmid, pSV2Cdhfr (Fig. 1), into a dihydrofolate reductase-negative line of CHO cells (7). This plasmid consists of the entire structural region of the bovine growth hormone gene driven by the immediate early promoter of the human cytomegalovirus. The construct is inserted into a plasmid (pSV2dhfr) containing the dihydrofolate reductase gene, which is amplified by growing the cells in the presence of 3 μM methotrexate. This cell line synthesizes and secretes abundant quantities of bovine growth hormone, and we estimate that bovine growth hormone mRNA accounts for approximately 5% of the total poly(A)$^+$ RNA in these cells. Nuclear and cytosolic RNA were isolated from CHO 14-10-4 cells and analyzed by blot hybridization using a cDNA probe and probes specific for each of the four introns (Fig. 2). As expected, species of poly(A)$^+$ RNA hybridizing to each of the intron probes were detected in the nuclear RNA fraction, presumably representing precursors of mature bovine growth hormone mRNA. Unexpectedly, a distinct RNA was detected in the cytoplasmic RNA that hybridized to the probe specific for intron D. Furthermore, the amount of this RNA in the nuclear RNA fraction was significantly greater than any of the other intron-containing RNAs. RNAs hybridizing to any of the other three intron-specific probes were not detectable in the cytosolic RNA fraction.

The size of the cytoplasmic RNA hybridizing to the intron D-specific probe was consistent with a bovine growth hormone mRNA from which intron D has not been removed from an otherwise normal growth hormone mRNA (approximately 1300 nucleotides for the variant vs. approximately 1000 nucleotides for wild-type mRNA). To determine that intron D is contiguous with exon 4 and exon 5 in this variant mRNA, CHO 14-10-4 polysomal poly(A)$^+$ RNA was analyzed by nuclease S1 mapping (Fig. 3). The hybridization probe was a 1198-nucleotide genomic fragment extending from a *Sac* II site in the middle of intron C to an *Eco*RI site 426 nucleotides downstream from the polyadenylylation site.

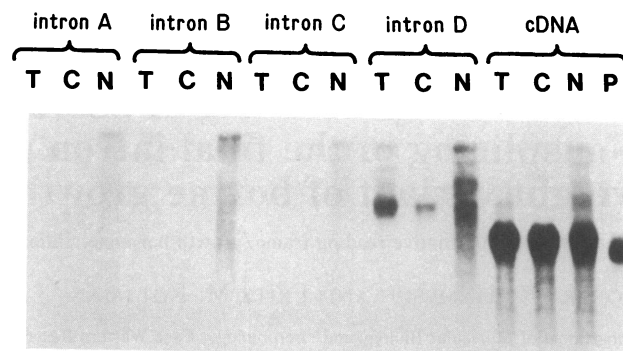


FIG. 2. Blot-hybridization analysis of RNA isolated from CHO 14-10-4 cells. Total (lanes T), cytosolic (lanes C), and nuclear (lanes N) poly(A)$^+$ RNA was isolated and analyzed by RNA blots with probes specific for each of the four introns and a cDNA probe of bovine growth hormone. All probes were of approximately equal specific activity. The probe for intron A is a 126-base *Alu* I/*Alu* I fragment spanning from 78 nucleotides past the end of exon 1 to 34 nucleotides from the start of exon 2. The intron B-specific probe is a 192-base *Alu* I/*Alu* I fragment starting 5 nucleotides past the end of exon 2 and extending to 32 nucleotides from the start of exon 3. The probe specific for intron C is a 176-nucleotide *Alu* I/*Alu* I fragment starting 62 bases past the end of exon 3 and extending 9 bases into exon 4. The intron D-specific probe is a 202-base *Hae* III/*Hae* III fragment extending from 38 nucleotides past the end of exon 4 to within 35 nucleotides of the start of exon 5. The cDNA probe is a 320-nucleotide *Pst* I/*Pst* I fragment located at the 5′ end of the gene. P, pituitary poly(A)$^+$ RNA.

The results of this nuclease S1 cleavage-protection experiment are consistent with the existence of a bovine growth hormone mRNA from which intron D has not been removed. If intron D were not removed, a fragment 739 nucleotides long would be protected upon digestion with nuclease S1, which was indicated by the protected fragment approximately 750 nucleotides in length. Wild-type bovine growth hormone mRNA, which lacks intron D, will result in protection of two fragments 162 and 303 nucleotides long, corresponding to exons 4 and 5, respectively. The intensity of the protected fragment ascribed to a RNA sequence containing intron D, as compared to the bands at 162 and 303 nucleotides, is consistent with the relative amount of alternatively processed

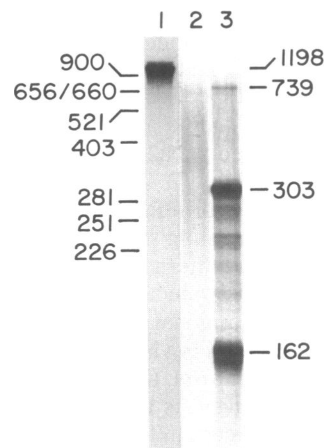
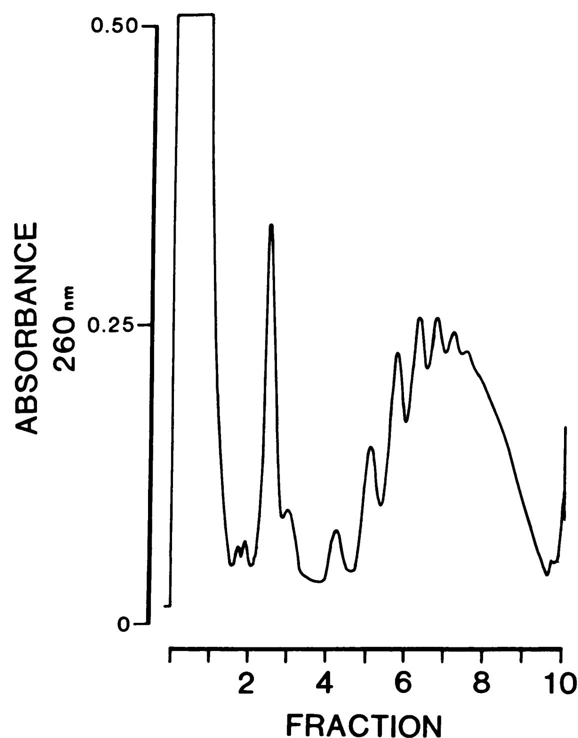


FIG. 3. Nuclease S1 analysis of CHO 14-10-4 polysomal poly(A)$^+$ RNA. Polysomal poly(A)$^+$ RNA (250 ng) from CHO 14-10-4 cells was hybridized to a uniformly labeled, single-stranded probe spanning exon 4, intron D, and exon 5. After digestion with S1 nuclease (100 Vogt units for 1 hr), protected fragments were analyzed on a 6% polyacrylamide sequencing-type gel. Lines indicate the position of size markers (shown as $M_r \times 10^{-3}$; pBR322 digested with *Alu* I). Lanes: 1, untreated probe; 2, probe mock-hybridized with 1 g of tRNA; 3, CHO 14-10-4 polysomal poly(A)$^+$ RNA.

mRNA observed in these cells by blot-hybridization analysis. The identity of additional bands between 303 and 162 nucleotides in length is unknown.

The observation that this intron D-containing RNA is the only intron-containing RNA detected in both the nucleus and the cytoplasm suggests its presence in the cytoplasm is probably not the result of leakage of mRNA precursors from the nucleus during preparation of the nuclear and cytosol fractions. To further verify this result and determine if this variant bovine growth hormone mRNA is being translated into a polypeptide, polysomal fractions of CHO 14-10-4 cells were separated on sucrose density gradients, and the fractions were analyzed for the intron D-containing mRNA (Fig. 4). The intron D-containing mRNA was detected across the polysomal profile in parallel with the wild-type growth hormone mRNA, suggesting this variant mRNA is being translated into a bovine growth hormone-related polypeptide. Further verification of the location of this RNA on



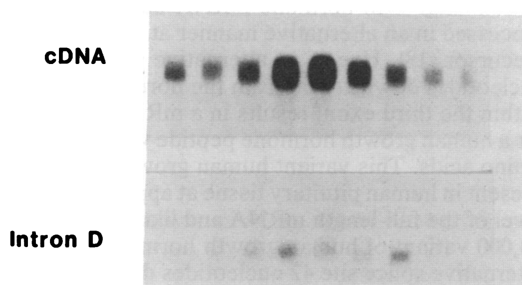


FIG. 4. Blot-hybridization analysis of polyadenylylated RNA prepared from polysomes isolated from CHO 14-10-4 cells. Fifteen A_{260} units of a cytosolic extract (0.3 ml) was sedimented through a 15–40% linear sucrose density gradient. The gradient was fractionated, and the absorbance was monitored at 260 nm with an Isco gradient fractionator (sedimentation is left to right). The total volume of the gradient was 12 ml; 10 equal fractions were collected and analyzed with a cDNA probe and a probe specific for intron D. The membrane that hybridized with the cDNA probe was exposed for 24 hr without an intensifying screen, and the intron D membrane was exposed for 4 days with an intensifying screen.

polysomes was demonstrated by treating the extracts with 10 mM EDTA to dissociate the ribosomes prior to centrifugation. Under these conditions the intron D-containing mRNA was located at the top of the gradient, ruling out the possibility that this RNA species is part of a large undefined ribonucleoprotein complex (data not shown).

Inspection of the nucleotide sequence of this variant mRNA revealed the presence of an open reading frame continuing from exon 4 and extending across the entire length of intron D with no termination codon encountered until 50 nucleotides into exon 5 (Fig. 5). It should be noted that exon 5 is shifted from the normal reading frame in this variant mRNA species. Translation of this intron D-containing species of bovine growth hormone mRNA predicts a protein that has 125 amino-terminal amino acids of wild-type growth hormone joined to 108 amino acids encoded by the 274 nucleotides of intron D along with the first 50 nucleotides of exon 5. Exon 5 normally would encode an additional 66 carboxyl-terminal amino acids. Hence, this growth hormone-related polypeptide would be 42 amino acids longer than the wild-type growth hormone or approximately 5000 greater in molecular weight.

The detection of the intron D-containing bovine growth hormone mRNA in the CHO 14-10-4 cell line and the presence of the open reading frame suggested the possibility that a similar variant mRNA might be present in pituitary tissue. To address this possibility, polysomal preparations from pituitary tissue were fractionated and analyzed for intron D-containing RNA. The preparation of intact polysomes from fresh bovine pituitary tissue was not possible because of the unavoidable length of time (10–15 min minimum) from sacrifice of the animal to obtaining the tissue, along with the necessity of adding an inhibitor of elongation to prevent ribosome run-off. This problem was circumvented by utilizing pituitary tissue slices to prepare polysomal fractions. As with the CHO 14-10-4 cells, the intron D-containing species of growth hormone mRNA was detected across the entire polysomal profile, indicating that this alternatively processed mRNA also is expressed in pituitary tissue (Fig. 6). Southern blot analysis has suggested previously that there is a single copy of the bovine growth hormone gene (2). Southern analysis of bovine DNA with the probe specific for intron D resulted in a pattern identical to that obtained with a cDNA probe (data not shown), providing evidence that both the wild-type and variant growth hormone mRNAs are transcribed from a single gene in pituitary tissue.

DISCUSSION

We have demonstrated the existence of a variant bovine growth hormone mRNA from which the final intron is not removed by splicing. This intron D-containing variant of bovine growth hormone mRNA can be detected in a stably transfected cell line as well as in anterior pituitary tissue. The fact that this mRNA can be detected in pituitary tissue suggests that this variant mRNA could be physiologically significant. Precedent for this type of alternative processing is provided by the γ-fibrinogen gene in which the seventh and final intron is not removed from approximately 10% of the precursor mRNAs (14).

The nonsplicing of intron D from bovine growth hormone mRNA implies that the initial region of exon 5 is expressed in more than one reading frame. The use of multiple reading frames appears to be rare with eukaryotic cellular mRNAs, although it has been demonstrated for numerous viral sequences (15). Human gastrin-releasing peptide is one example where a eukaryotic DNA sequence encoding a cellular mRNA is apparently read in more than one reading frame as a result of alternative mRNA processing (16). However, it does not alter the 28 amino acids that constitute the biolog-

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125                 130                                          140
Arg Val Gly Met Ala Leu Trp Val Pro Ser Met Leu Gly Ala Met Pro Ala Leu Ser
CCG gtg ggg atg gcg ttg tgg gtc cct tcc atg ctg ggg gcc atg ccc gcc ctc tcc

                        150                                          160
Trp Leu Ser Gln Glu Asn Ala Arg Gly Leu Gly Glu Thr Asp Pro Cys Ser Leu Pro
tgg ctt agc cag gag aat gca cgt ggg ctt ggg gag aca gat ccc tgc tct ctc cct

                                170                                  180
Leu Ser Ser Ser Pro Ala Leu Thr Gln Gly Lys Pro Phe Pro Leu Leu Lys Pro Pro
ctt tct agc agt cca gcc ttg acc cag ggg aaa cct ttt ccc ctt tgg aaa cct cct

                                    190                                  200
Ser Ser Pro Phe Ser Lys Pro Val Gly Glu Gly Gly Lys Trp Ser Gly Gln Glu Gly
tcc tcg ccc ttc tcc aag cct gta ggg gag ggt gga aaa tgg agc ggg cag gag gca

                                        210
Ala Ala Pro Glu Gly Pro Ser Ala Ser Leu Ser Leu Pro Pro Leu Ala Gly Ala Gly
gct gct cct gag ggc cct tcg gcc tct ctg tct ctc cct ccc ttg gca gGA GCT GCA
                                                                Glu Leu Glu
220                                 230
Arg Trp His Pro Pro Gly Trp Ala Asp Pro Gln Ala Asp Leu  *
AGA TGG CAC CCC CCG GGC TGG GCA GAT CCT CAA GCA GAC CTA TGA CAA ATT TGA CAC
 Asp Gly Thr Pro Arg Ala Gly Gln Ile Leu Lys Gln Thr Tyr Asp Lys Phe Asp Thr
     130                                    140

AAA CAT GCG CAG TGA CGA CGC GCT GCT CAA GAA CTA CGC TCT GCT CTC CTG CTT CCG
 Asn Met Arg Ser Asp Asp Ala Leu Leu Lys Asn Tyr Gly Leu Leu Ser Cys Phe Arg
         150                                    160

GAA CGA CCT GCA TAA GAC GGA GAC GTA CCT GAG GGT CAT GAA GTG CCG CCG CTT CGG
 Lys Asp Leu His Lys Thr Glu Thr Tyr Leu Arg Val Met Lys Cys Arg Arg Phe Gly
             170                                    180

GGA GGC CAG CTG TGC CTT CTA Gttgccagccatctgttgtttgcccctcccccgtgccttccttgaccc
 Glu Ala Ser Cys Ala Phe  *
                 190

tggaaggtgccactcccactgtcctttcctaataaaatgaggaaattgcatcgc(A)n
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FIG. 5. Amino acid translation of the nucleotide sequence of the variant region of the intron D-containing bovine growth hormone mRNA. Intron sequences and the 3′ flanking region of wild-type growth hormone are presented in lower-case letters, with exon sequences in uppercase letters. Translation of the intron D-containing variant is printed in bold letters above the sequence, with the normal translation of exon 5 printed below the sequence. The CCG arginine codon located at the beginning of the sequence is the final codon of exon 4. Numbers indicate the amino acid position in the polypeptide with position 1 starting after the signal peptide.

ically active gastrin-releasing peptide that results from processing of the prohormone.

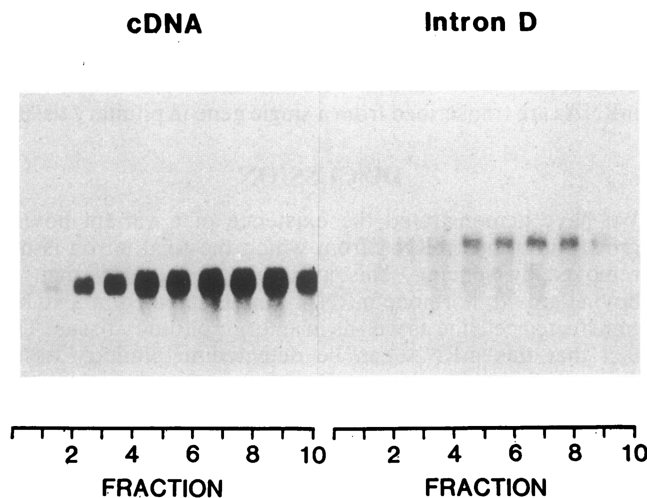

FIG. 6. Blot-hybridization analysis of polysomal RNA fractions isolated from bovine anterior pituitary slices. Cytoplasmic extracts were prepared from slices of bovine pituitary tissue as described, and 15 A_{260} units of the extract was fractionated on sucrose density gradients and analyzed for wild-type and intron D-containing RNAs. The lanes hybridized with the probe specific for intron D contained 80% of the sample, and the membrane was exposed for 5 days with an intensifying screen. The remaining 20% was analyzed with the cDNA probe, in which case the membrane was exposed for 20 hr without an intensifying screen.

Whether growth hormone mRNA from other species is alternatively processed in a manner analogous to bovine growth hormone mRNA remains to be determined. It has been noted previously that there exists a striking degree of homology between intron D of the human growth hormone gene and intron D of the bovine gene (17). Examination of nucleotide sequence of intron D of the human growth hormone gene also reveals the presence of an open reading frame through intron D of the human gene with a stop codon encountered at the very end of the intron.

Human growth hormone mRNA has been reported to be processed in an alternative manner at another site within the precursor (18). Use of an alternative splice acceptor site 45 nucleotides downstream from the normal 3′ end of intron B, within the third exon, results in a mRNA species that codes for a human growth hormone peptide with 15 deleted internal amino acids. This variant human growth hormone mRNA is present in human pituitary tissue at approximately 10% of the level of the full-length mRNA and likely accounts for the M_r 20,000 variant of human growth hormone (1). An analogous alternative splice site 42 nucleotides downstream from the 3′ end of intron B exists in the bovine growth hormone gene (2). However, using nuclease S1 mapping, we have been unable to obtain any evidence for the use of this alternative splice acceptor site in bovine growth hormone mRNA (unpublished observations).

The physiological significance of a variant bovine growth hormone with a modified carboxyl terminus remains unknown. It has been estimated previously that bovine growth hormone mRNA accounts for 10% of the total mRNA in the anterior pituitary (19, 20), and bovine growth hormone-producing cells make up only a fraction of the total cell types

in the anterior pituitary (21). We estimate the intron D-containing mRNA is present at approximately 0.1% of the wild-type mRNA, corresponding to 0.01% of the total mRNA in the anterior pituitary. Hence, assuming both the intron D-containing and wild-type mRNA are expressed in the same cell, the variant mRNA is present at a significant level.

Several aspects of the amino acid sequence of the variant region of the predicted polypeptide arising from this mRNA are worth noting. First, this region of the molecule is unusually rich in proline and serine, containing 18 proline and 14 serine residues out of a total of 108 amino acids. Second, there is a relatively hydrophobic stretch of 24 amino acids at the start of this variant region, delineated by an arginine at the end of exon 4 and a glutamic acid at amino acid residue 148, raising the possibility that this polypeptide might be located in a membrane. Third, in wild-type growth hormone, there exists a disulfide bond between cysteine-53 and cysteine-164 (22). Cysteine-164 would be eliminated in a polypeptide derived from the intron D-containing mRNA. There is, however, a cysteine near the same position (cysteine-159) in the variant polypeptide. Whether this cysteine residue is part of a disulfide bond with cysteine-53 remains to be determined. Finally, the possibility also must be considered that the polypeptide could be further modified or processed in some manner.

In conclusion, the composition of the carboxyl-terminal region of the bovine growth hormone-related polypeptide that would be derived from this variant mRNA predicts a protein with properties differing significantly from wild-type bovine growth hormone, perhaps involving a subcellular location that differs from wild-type growth hormone. These possibilities along with the determination of the physiological fate of this variant protein must await the development of antibodies specific to the unique carboxyl portion of the variant bovine growth hormone.

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