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Requirement for the 3′ flanking region of the bovine growth hormone gene for accurate polyadenylylation

(eukaryotic RNA processing/poly(A) signal)

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ABSTRACT **We examined whether the sequence extending 3′ to the polyadenylylation site of the bovine growth hormone gene contains any signal that affects the polyadenylylation of the growth hormone mRNA. For this purpose, cloned copies of this gene, each containing a different length of growth hormone-specific sequence 3′ to the wild-type polyadenylylation site, were used to transfect COS-1 cells. The polyadenylylation site on the mRNAs produced from the exogenously added growth hormone genes were analyzed with an S1 nuclease mapping procedure. We found that a gene containing 84 base pairs of its own 3′ flanking sequence is capable of producing an accurately polyadenylylated mRNA. On the other hand, genes containing only 1, 10, or 13 base pairs of 3′ flanking sequence were principally polyadenylylated at discrete sites either upstream or downstream from the wild-type position. Using a computer program, we examined whether secondary structures on the primary growth hormone transcript correlated with the site where the mRNA is polyadenylylated.**

All of the specific signals that are used to direct the location and efficiency of polyadenylylation of an mRNA molecule have not been thoroughly characterized. Most higher eukaryotic genes coding for polyadenylylated mRNAs contain the sequence A-A-T-A-A-A about 10–30 nucleotides upstream from their polyadenylylation site (1). This evolutionarily conserved hexanucleotide has been determined to be essential for the production of polyadenylylated mRNAs (2, 3), and is an important signal for efficient processing of the 3′ terminus of the primary RNA transcript (4, 5). However, this sequence cannot be the only signal that regulates the polyadenylylation reaction because some genes contain additional A-A-T-A-A-A hexanucleotides that do not appear to function as polyadenylylation signals (6–8).

We wanted to determine whether the 3′ flanking sequence of the bovine growth hormone gene contains any signal that, in addition to the A-A-T-A-A-A, may function to regulate the polyadenylylation reaction. For this purpose, several different cloned copies of this gene, each containing a different length of growth hormone-specific 3′ flanking sequence, were used to transfect COS-1 monkey cells (9). Our results show that a growth hormone gene containing 84 base pairs (bp) of its own 3′ flanking sequence is capable of expressing an accurately polyadenylylated mRNA. Conversely, genes containing only 1, 10, or 13 nucleotides of growth hormone-specific sequence beyond the polyadenylylation site produced mRNAs that were mainly polyadenylylated at sites upstream or downstream from the wild-type position. From these results it appears that the bovine growth hormone gene contains a signal within its first 84 bp of 3′ flanking sequence that influences where the growth hormone mRNA is polyadenylylated.

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METHODS AND MATERIALS

Cell Culture and Transfection Conditions. COS-1 cells were provided by Y. Gluzman (Cold Spring Harbor Laboratory) and were grown and transfected with the expression plasmids as described by Mellon *et al.* (10).

Construction of the Expression Plasmids. The transient expression plasmid pSVB3/Ba (Fig. 1) contains a "poison-minus" (11) deletion mutant of pBR322, pBRH (unpublished results), and the 400-bp *Hpa* II/*Hin*dIII fragment from simian virus 40 (SV40) that contains the SV40 replication origin, the 72-bp repeat enhancer sequence (12), and the early and late region SV40 promoters. The SV40 *Hpa* II site was converted into a *Bam*HI site in the vector. A 2.2 kilobase (kb) *Bam*HI/*Eco*RI fragment, containing the entire coding region and the first 400 bp of 3′ flanking sequence of the bovine growth hormone gene (13), was used in the construction of the pSVB3/Ba plasmid. The growth hormone gene on this restriction fragment lacks its own promoter and is expressed from the late region SV40 promoter on the pSVB3/Ba plasmid. A series of deletion mutants of the growth hormone gene, containing various lengths of growth hormone-specific 3′ flanking sequence, were prepared by resection with BAL-31 nuclease from the *Eco*RI site (Fig. 1). The exact amount of growth hormone-specific 3′ flanking sequence associated with these plasmids was determined by sequencing the DNA, by utilizing the dideoxy chain terminator procedure (14).

RNA Preparation and Analysis. Cytoplasmic RNA was prepared with the vanadium ribonucleoside ribonuclease inhibitor (15, 16). Poly(A)$^+$ RNA was prepared by using oligo(dT) columns (16). The S1 nuclease mapping procedure described by Weaver and Weissmann (17) was used to analyze the polyadenylylation site on the mRNA.

RESULTS

Expression of the Growth Hormone Gene in COS-1 Cells. We chose to study the polyadenylylation reaction by expressing cloned copies of the bovine growth hormone gene in mammalian cells. Our previous characterization of this gene revealed that it is approximately 1800 bp long and contains four intervening sequences (13). A 2.2-kb *Bam*HI/*Eco*RI restriction fragment containing the entire growth hormone gene along with 400 bp of its 3′ flanking sequence was used to construct the pSVB3/Ba expression plasmid (Fig. 1).

After the transfection of the pSVB3/Ba plasmid into COS-1 cells, blot hybridization was used to analyze poly(A)$^+$ cytoplasmic RNA for the presence of bovine growth hormone transcripts. Comparison of the level of the bovine growth hormone mRNA with the endogenous α-tubulin mRNA in COS-1 cells led us to estimate that the growth hormone transcripts represented about 5% of the poly(A)$^+$ RNA in these

Abbreviations: bp, base pair(s); kb, kilobase pair(s); SV40, simian virus 40.

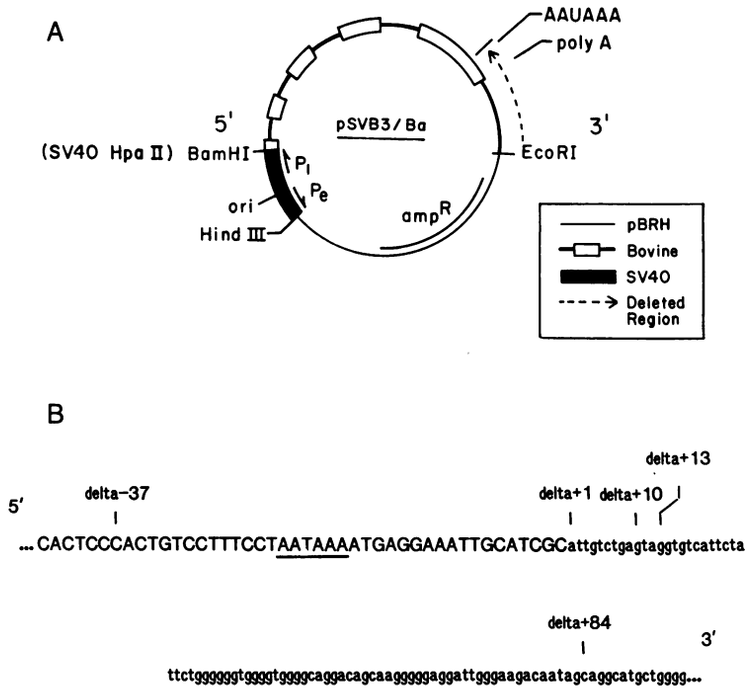

FIG. 1. (*A*) Structure of the expression plasmid pSVB3/Ba. The 2.2-kb *Bam*HI/*Eco*RI DNA fragment containing the entire coding region of the bovine growth hormone gene along with its first 400 bp of 3′ flanking sequence is positioned downstream from the SV40 sequence containing the SV40 replication origin (*ori*), 72-bp repeat enhancer element, and the early and late region promoters (P_e and P_l). The pBRH sequence is a "poison-minus" (11) deletion derivative of pBR322 that we prepared (unpublished results). The portion of the pSVB3/Ba plasmid that was resected with BAL-31 to produce the growth hormone deletion mutants is shown with a dashed arrow. amp^R, ampicillin resistance. (*B*) Sequence of the growth hormone gene near its wild-type polyadenylylation site. The 3′ untranslated sequence is shown in capital letters, and the lowercase letters represent the 3′ flanking sequence of the gene. The amount of 3′ flanking sequence associated with the delta−37, delta+1, delta+10, delta+13, and delta+84 deletion mutants is indicated.

cells (data not shown). Furthermore, immunologically active bovine growth hormone was synthesized in these cells and was secreted into the growth medium (data not shown).

Analysis of the Polyadenylylation Site on the mRNAs Produced from the pSVB3/Ba Plasmid. An S1 nuclease mapping procedure (17) was used to establish that oligo(dT)-selected growth hormone mRNA produced from the pSVB3/Ba plasmid in COS-1 cells was accurately polyadenylylated. The 700-bp *Sma* I/*Eco*RI genomic restriction fragment, which spans the growth hormone polyadenylylation site (Fig. 2*A*), was used as a probe for this experiment. The pituitary growth hormone mRNA protected a DNA fragment that was estimated to be 281 nucleotides long (Fig. 2*B*). This result is in agreement with the distance between the *Sma* I recognition sequence and the C-A dinucleotide that is located at the polyadenylylation site in the full-length growth hormone cDNA clone pG23 (13). Most importantly, the result obtained with the growth hormone mRNA produced by the pSVB3/Ba plasmid in COS-1 cells was indistinguishable from that obtained with the bovine pituitary RNA (Fig. 2*B*). This indicates that all of the signals that are necessary for the production of an accurately polyadenylylated mRNA are associated with the growth hormone gene on the pSVB3/Ba plasmid.

Analysis of the Polyadenylylation Site on the Growth Hormone mRNA Produced by the Deletion Mutants of the Growth Hormone Gene. Since the growth hormone sequence on the pSVB3/Ba plasmid is transcribed into mRNA that is polyadenylylated at the correct site, we wanted to determine whether some or all of the 400 bp of 3′ flanking sequence on this plasmid is required for accurate polyadenylylation of the mRNA. For this purpose, a series of deletion mutants of the growth hormone gene was prepared that contain different lengths of the growth hormone 3′ flanking sequence (Fig. 1). The delta+1, delta+10, delta+13, and delta+84 plasmids were derived from the pSVB3/Ba molecule and contain only the first 1, 10, 13, and 84 bp of growth hormone 3′ flanking sequence, respectively (Fig. 1*B*). The delta−37 gene contains no growth hormone 3′ flanking sequence and, furthermore, lacks the 37 bp of its 3′ untranslated region, which contains the hexanucleotide A-A-T-A-A-A (Fig. 1*B*).

The plasmids containing the deletion mutants of the growth hormone gene were used to transfect COS-1 cells and the polyadenylylation sites on the mRNAs that were produced were analyzed with the same S1 nuclease mapping procedure used above (17). In this experiment, however, the *Pvu* II site was used instead of the *Sma* I site to prepare the 3′-end-labeled DNA fragments (Fig. 3*A*). Also, because the sequences of the different resected growth hormone genes diverged near the wild-type polyadenylylation site, it was necessary to prepare three different 3′-end-labeled DNA probes for this analysis (Fig. 3*A*).

The pituitary growth hormone mRNA and the polyadenylylated growth hormone transcripts produced from the transient expression of the pSVB3/Ba and delta+84 genes all protected an identically sized DNA fragment that was estimated to be 115 nucleotides in length (Fig. 3*B*). This result is in agreement with the distance between the *Pvu* II site and the wild-type polyadenylylation site on the gene. The minor heterogeneity in the length of the protected DNA fragment is most likely an artifact associated with using the *Pvu* II/*Pst* I labeled DNA genomic fragment as a probe for this analysis; we cannot, however, rule out that the mRNA molecules themselves are slightly heterogeneous at their 3′ termini. From these results it appears that a growth hormone gene containing only 84 bp of its own flanking sequence is still capable of producing an mRNA that is polyadenylylated at the wild-type site.

Unlike the transcript of the delta+84 gene, the RNA transcript produced from the delta−37 gene was not accurately polyadenylylated; it protected a DNA fragment that was estimated to be 278 nucleotides long (Fig. 3*B*, lane a). Protection of this sized DNA fragment indicates that the mRNA produced by this gene is polyadenylylated at a specific site in the pBR322 sequence that flanks the gene on its 3′ side (Fig. 3*C*). This result with the delta−37 gene is not unexpected, since this gene does not contain the evolutionarily conserved A-A-T-A-A-A hexanucleotide within its 3′ untranslated region.

The delta+1 gene produced polyadenylylated growth hormone mRNAs that protected DNA fragments that were estimated to be 110, 118, 140, and 315 nucleotides long (Fig. 3*B*, lane b). Additionally, the majority of transcripts expressed from the delta+10 and delta+13 genes protected a DNA fragment that was 110 nucleotides in length (Fig. 3*B*, lanes c and d). Some of the mRNAs produced by the delta+10 and

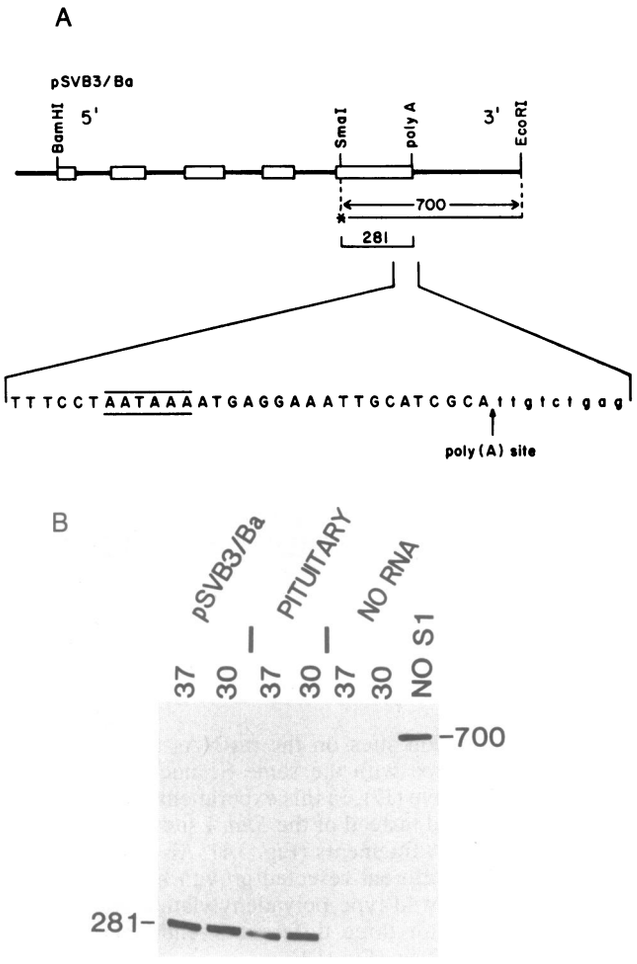

FIG. 2. S1 nuclease mapping of the 3' termini of the growth hormone mRNAs from a bovine pituitary and from COS-1 cells transfected with the pSVB3/Ba plasmid. (*A*) Structure of the 3'-end-labeled DNA fragment that was used as a probe for this experiment. The number below the fragment refers to the size of the DNA fragment protected by the mRNA. The nucleotide sequence of the growth hormone gene near its polyadenylylation site is also shown. The A-A-T-A-A-A sequence, within the 3' untranslated region, is underlined, and the polyadenylylation site, corresponding to the size of the protected DNA fragment, is indicated with an arrow. The 3' untranslated sequence is presented in capital letters, while the 3' flanking sequence is shown in lower-case letters. The occurrence of an A residue at the polyadenylylation site made it impossible to assign the wild-type polyadenylylation site specifically to the C or A nucleotides on the sequence. (*B*) Autoradiogram of a gel showing the DNA fragment protected by 1/8 of the polyadenylylated RNA prepared from a 175-cm^2 tissue culture flask of COS-1 cells transfected with the pSVB3/Ba plasmid or 1 μg of polyadenylylated RNA prepared from a bovine pituitary. As controls, the 3'-end-labeled DNA fragment was subjected to the hybridization conditions in the absence of any RNA or was analyzed without the S1 nuclease treatment. The number 281 refers to the size of the DNA fragment protected by the growth hormone mRNA. Each sample was digested with S1 nuclease at both 30°C and 37°C.

delta+13 genes also protected a 115-nucleotide fragment (Fig. 3*B*, lanes c and d). These results clearly demonstrate that growth hormone genes containing only their first 1, 10, or 13 nucleotides of the growth hormone 3' flanking sequence produce mRNAs that are principally polyadenylylated at discrete sites either upstream or downstream from the wild-type position.

DISCUSSION

We utilized a DNA-mediated gene transfer procedure to express deletion mutants of the bovine hormone gene in COS-1 cells. The genes that were used for this purpose contain different lengths of growth-hormone-specific 3'-flanking sequence. A gene containing 84 bp of 3'-flanking sequence, delta+84, was capable of producing an accurately polyadenylylated mRNA. On the other hand, the delta−37, delta+1, delta+10, and delta+13 growth hormone genes, containing 0, 1, 10, and 13 nucleotides of growth hormone 3'-flanking sequence, produced mRNAs that were principally polyadenylylated at sites upstream or downstream from the wild-type position. We estimated that the primary upstream polyadenylylation site was at a C-A dinucleotide that is 5 nucleotides away from the wild-type position (Fig. 3*C*). The most commonly used downstream polyadenylylation site was estimated to be at a specific site within the pBR322 sequence that flanks the growth hormone gene on its 3' side (Fig. 3*C*). From these results it appears that the bovine growth hormone gene contains some signal within its first 84 bp of 3' flanking sequence that is capable of influencing where the growth hormone mRNA will be polyadenylylated.

Concurrent with the experiments described here, Simonsen and Levinson (18) studied the processing and polyadenylylation signals of the hepatitis B virus surface antigen (HBsAg) gene. They found that some of the sequence beyond the polyadenylylation site of this gene was necessary for the efficient polyadenylylation of the HBsAg mRNA at the correct site. Their results are in agreement with those reported here for the bovine growth hormone gene; both the HBsAg and bovine growth hormone genes appear to have some signal within their 3' flanking region that affects the polyadenylylation reaction. Additionally, Birchmeier *et al.* (19) found that a region of sequence to the 3' side of the histone H2A gene, which codes for a nonpolyadenylylated mRNA, was necessary for the efficient formation of genuine H2A mRNA.

We are presently uncertain as to how the 3' flanking sequence of the growth hormone gene affects the polyadenylylation reaction. Several eukaryotic genes have been shown to be transcribed beyond their polyadenylylation site (20–26). If this is also the case for the growth hormone gene, the primary growth hormone transcript would have to be processed, possibly by a specialized endonuclease, before the poly(A) tail can be added onto the molecule. Recently it was reported that the highly conserved A-A-U-A-A-A hexanucleotide within the 3' untranslated region appears to be involved in regulating the 3' end processing of the primary transcript (2, 3). Our results indicate that a signal within the 3' flanking region of the primary growth hormone transcript is functional in directing precisely where this processing occurs. Specific nucleotides downstream from the polyadenylylation site may serve as part of the recognition sequence for the processing enzyme. Alternatively, the 3' flanking sequence on the primary RNA transcript may form a specific secondary structure that somehow affects where the molecule will be processed. To determine whether RNA secondary structures could form near the polyadenylylation site on the primary transcript from the wild-type and deletion mutants of the growth hormone gene, the sequences near the 3' end of these genes were subjected to computer analysis (Los Alamos National Laboratories programs). Fig. 4 shows possible conformations for the primary transcripts of the wild-type, delta+84, delta+1, delta+10, and delta+13 genes. Both the wild-type sequence and the deletion clone delta+84 are capable of forming the stem-and-loop shown in Fig. 4*A*. This thermodynamically stable structure includes 100 bases of the 3' untranslated region and a considerable amount of the 3' flanking sequence. In contrast, a potential secondary structure of the transcript from the delta+1 gene is shown in Fig. 4*B*; this structure is substantially less stable than the structure that is shown for the delta+84 transcript. The sequence just upstream from the polyadenylylation site in the

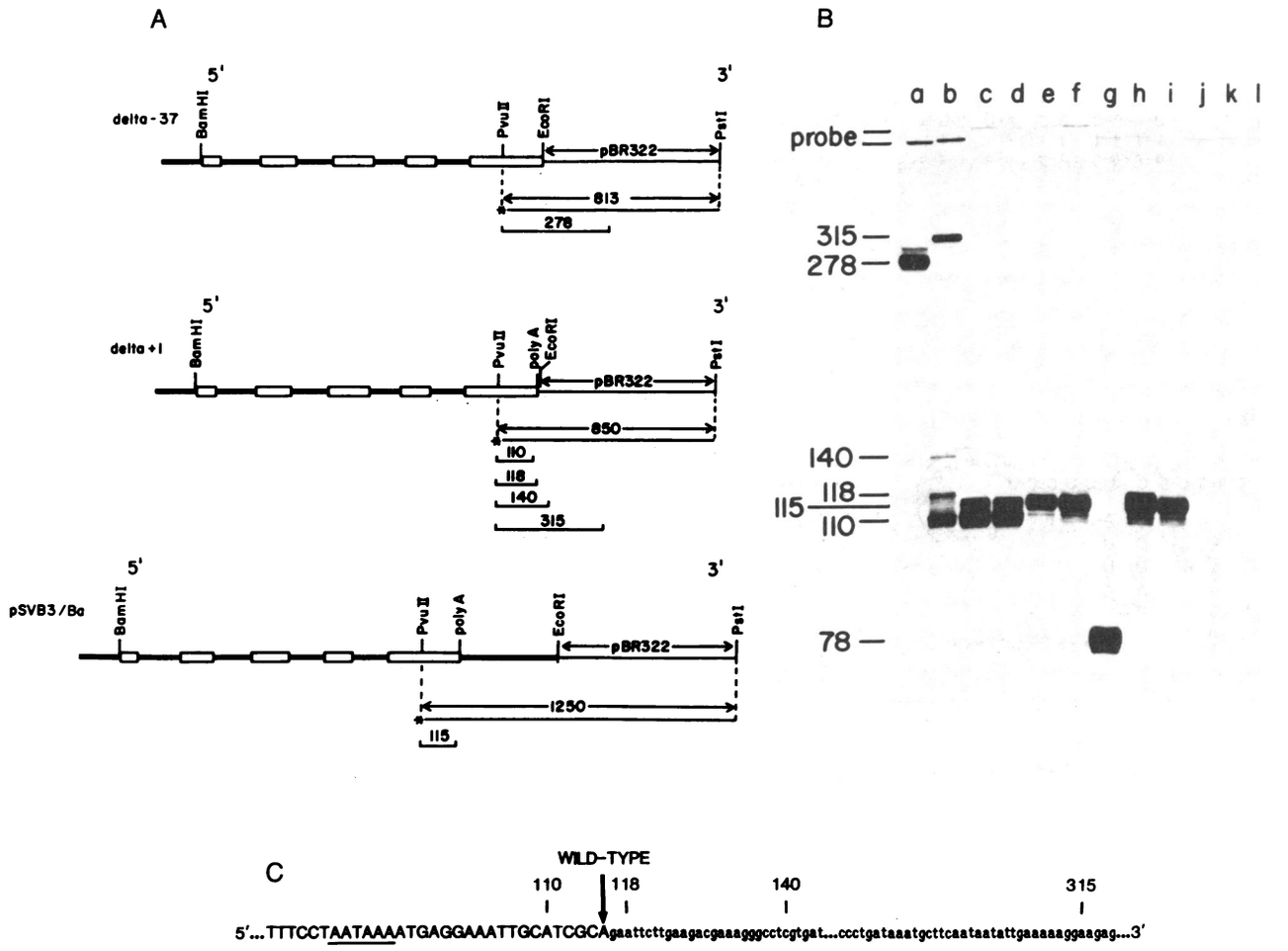

FIG. 3. S1 nuclease mapping of the 3′ termini of growth hormone mRNAs transcribed from the expression plasmids delta−37, delta+1, delta+10, delta+13, delta+84, and pSVB3/Ba. (*A*) Structure of the 3′-end-labeled DNA fragments used as probes for this experiment. In the expression plasmids delta−37 and delta+1, the growth hormone 3′ flanking sequence is replaced with a region of pBR322 at a point either upstream or near the wild-type polyadenylylation site; therefore, it was necessary to use the 813-bp and the 850-bp *Pvu* II/*Pst* I fragments from the delta−37 and delta+1 plasmids, respectively, to analyze the mRNA produced from the delta−37 and delta+1 genes. The corresponding 1250-bp *Pvu* II/*Pst* I fragment from the pSVB3/Ba plasmid was used as a probe to analyze the RNA produced from the other transfected genes. Numbers below each gene refer to the size of the DNA fragments protected by the mRNAs. (*B*) Autoradiogram of a gel showing the size of the DNA fragments protected by 1/8 of the polyadenylylated RNA extracted from a 175-cm^2 tissue culture flask of COS-1 cells transfected with the delta−37 (lane a), delta+1 (lane b), delta+10 (lane c), delta+13 (lane d), delta+84 (lane e), and pSVB3/Ba (lane f) expression plasmids. For controls, 250 ng of polyadenylylated RNA from a bovine pituitary was hybridized with the 3′-end-labeled DNA fragments prepared from the delta−37 (lane g), delta+1 (lane h), and pSVB3/Ba (lane i) plasmids. These controls were included to show the small degree of size heterogeneity of the protected DNA fragments, which appears to be an artifact of this kind of analysis. Lanes j–l are duplicates of lanes g–i, except that the RNA was omitted from the hybridization reaction. (*C*) Nucleotide sequence of the delta+1 gene, showing the upstream and downstream polyadenylylation sites on the mRNA molecules produced by the delta−37, delta+1, delta+10, and delta+13 genes. The growth hormone sequence is represented in capital letters. The pBR322 sequence and part of the *Eco*RI molecular linker are shown in lowercase letters. Numbers refer to the estimated size, in nucleotides, of the protected DNA fragments that were observed on the autoradiogram (see above). The A-A-T-A-A-A hexanucleotide sequence within the 3′ untranslated region of the growth hormone gene is underlined. The polyadenylylation sites shown are only estimates based on the calculated size of the protected DNA fragments.

pBR322 sequence that was observed with the delta−37 and delta+1 genes is capable of forming the structure shown in Fig. 4*C*; in this case an A+U-rich sequence may transiently form a short looped-out structure. Compared to the delta+1 gene, the 9–12 additional nucleotides of growth hormone 3′-flanking sequence on the delta+10 and delta+13, respectively, appear to have enhanced the strength of the primary upstream polyadenylylation signal because none of the transcripts from these genes were polyadenylylated at the downstream cryptic site in pBR322. This observation correlates with the increased thermodynamic stability of the stem-and-loop structures on the delta+10 and delta+13 (Fig. 4*D*) transcripts as compared with the secondary structure on the delta+1 transcript (Fig. 4*B*).

Although no evidence is presented here which indicates that these structures actually exist *in vivo*, we find a good correlation between the position of the stem-loop structures in Fig. 4 and the observed polyadenylylation sites. In all cases a sequence like the A-A-U-A-A-A hexanucleotide occupies a single-stranded hairpin loop that is present just upstream from a single-stranded region containing the polyadenylylation site (Fig. 4). Additionally, the thermodynamic stability of the secondary structure appears to correlate with the degree of polyadenylylation at a specific site (compare delta+1 and delta+13, Fig. 4 *B* and *D*). Further experiments utilizing a point mutagenesis approach to disrupt the computer-predicted secondary structures will be necessary to evaluate what physiological role, if any, these structures play in the processing of the growth hormone primary transcript. The extensive degree of secondary structure near the

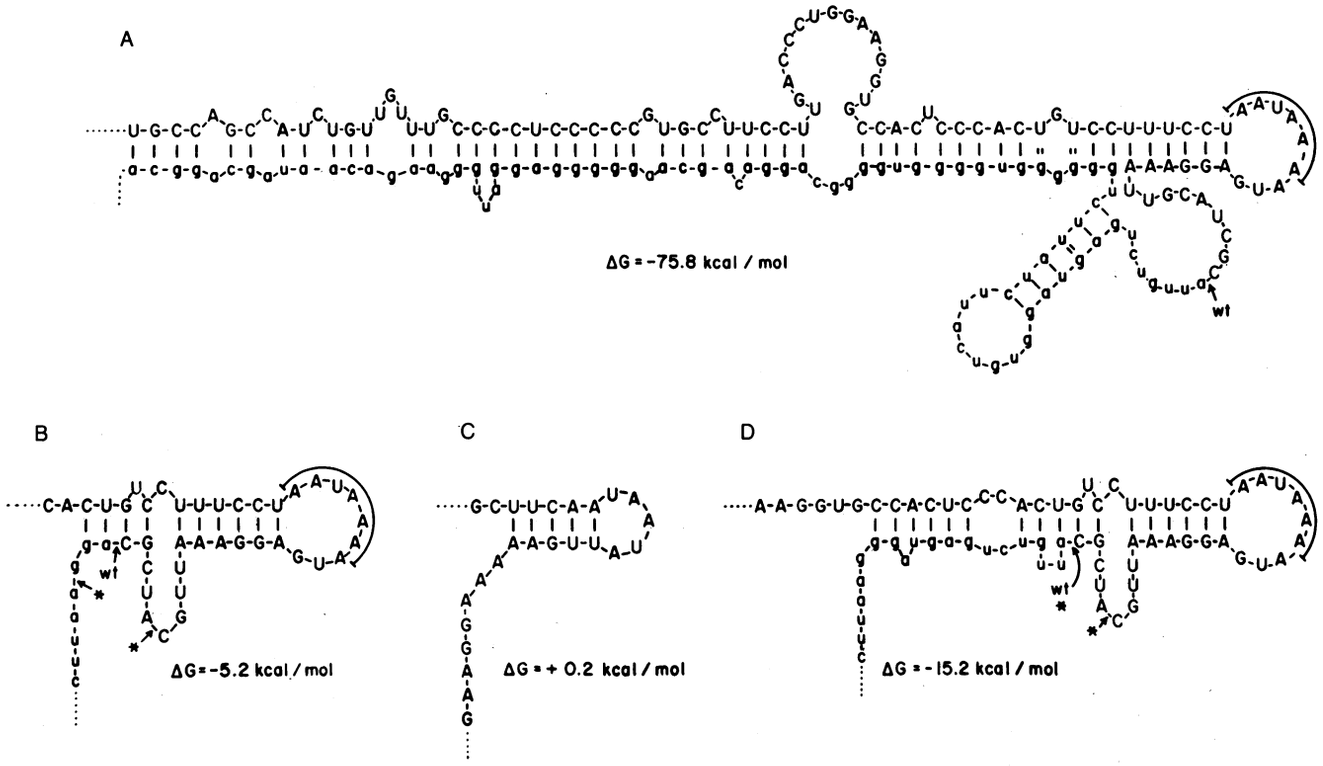

FIG. 4. Computer-generated secondary structures of the sequence near the 3′ end of the primary RNA transcripts produced from the wild-type and delta+84 genes (*A*) and the delta+1 (*B*) and delta+13 (*D*) genes. The sequence near the polyadenylylation site observed in the pBR322 sequence with the delta−37 and delta+1 genes was also subjected to this analysis (*C*). The bovine growth hormone wild-type (wt) polyadenylylation site is shown with an arrow. Polyadenylylation sites actually detected with each clone are indicated with asterisks. Clone delta+10 gives a structure almost identical to delta+13 with $\Delta G = -15.8$ kcal/mol (1 kcal = 4.184 kJ). In *B* and *D*, the 3′-terminal G in the duplex is contributed by the *Eco*RI linker. The conserved hexanucleotide A-A-U-A-A-A is outlined; the 3′ untranslated region is in capital letters, and the 3′ flanking sequence is in lowercase letters.

polyadenylylation site of the wild-type growth hormone gene may be associated with some posttranscriptional function that is characteristic of this gene and may not be a common feature of the primary transcripts produced from other eukaryotic genes.

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