The 3′-Flanking Sequence of the Bovine Growth Hormone Gene Contains Novel Elements Required for Efficient and Accurate Polyadenylation*

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In addition to the conserved AAUAAA hexanucleotide, GU- and U-rich sequences in the 3′-flanking region are thought to be critical for efficient polyadenylation. The 3′-flanking sequence requirements for efficient and accurate polyadenylation of the bovine growth hormone (bGH) gene were determined by quantitative S1 nuclease analysis of transcripts derived from various bGH 3′ deletions and block mutations transiently transfected into COS-1 cells. Though the bGH 3′-flanking sequence contains a portion of the putative GU efficiency element, we find that mutation of this element leads to a marginal decrease in efficiency similar to that from mutation of other sequences that do not contain recognizable GU- or U-rich motifs. The data are consistent with a diffuse efficiency element in the bGH polyadenylation signal rather than a discrete element as is thought to exist in other mammalian signals. We have also determined that a region from 18 to 27 nucleotides downstream of the cleavage site contains sequences required for correctly positioning the cleavage site.

Most eukaryotic pre-mRNAs are post-transcriptionally modified in the nucleus at their 3′ terminus by cleavage of the primary RNA transcript at a discrete site followed by the addition of approximately 200 adenylate residues to the 3′ end of the cleavage site (Darnell, 1982; Littauer and Soreq, 1982). The hexanucleotide AAUAAA, which is located some 10–30 nucleotides 5′ of the cleavage site in most polyadenylated messages is a critical component of the polyadenylation signal. Deletions or point mutations of this sequence cause a greater than 10-fold decrease in the efficiency of polyadenylation in vivo (Montell et al., 1983; Wickens and Stephenson, 1984) or in vitro (Wilusz et al., 1989; Sheets et al., 1990; Bardwell et al., 1991). The hexanucleotide also appears to be required for the efficient polymerization of the first 10 adenylates after the cleavage of the primary transcript (Sheets and Wickens, 1989).

In addition to this AAUAAA sequence, a general consensus has emerged that poorly conserved GU- and/or U-rich sequences located downstream of the cleavage site are critical for efficient cleavage (Birnstiel et al., 1985; McLauchlan et al., 1985; Munroe and Jacobson, 1990; Proudfoot, 1991). Perhaps the most convincing evidence supporting a central role for these sequences are experiments with the SV40 early, adenosine virus E2A, and rabbit β-globin polyadenylation signals demonstrating that efficient polyadenylation can be restored in constructs containing large deletions of 3′-flanking sequences by the insertion of oligonucleotides containing specific GU- and U-rich elements (McDevitt et al., 1986; Green and Hart, 1988; Levitt et al., 1989). Point mutations of the GU- and U-rich elements of the SV40 early and adenosine virus E2A polyadenylation signals have demonstrated that portions of these elements are rather sensitive to mutation and, surprisingly, that the efficiencies can be increased severalfold over the wild-type level by some of the sequence substitutions (McDevitt et al., 1986). Even though the downstream elements from different polyadenylation signals share little sequence identity, the observation that the downstream elements of the adenosine virus E2A polyadenylation signal can replace those of the heterologous SV40 early polyadenylation signal suggests a functional equivalence of downstream elements that transcends their apparent lack of homology (Hart et al., 1985).

The bovine growth hormone (bGH)1 gene does not possess an identifiable U-rich region and only contains a portion of the proposed GU-rich consensus sequence found in many polyadenylation signals. Thus, we chose to dissect the 3′-flanking region of the bGH gene with unidirectional deletions and site-directed mutations to identify the sequence elements responsible for efficient and accurate polyadenylation. We find that the sequences required for efficient bGH polyadenylation are present as a dispersed rather than discrete element that differs markedly from those described in other systems. We also identify a 9-nucleotide region 18 nucleotides downstream of the cleavage site that is required for accurate cleavage. The observed lack of a specific requirement for GU elements or for any discrete element in the 3′-flanking sequences suggests that the bGH polyadenylation signal represents a new paradigm of an efficient polyadenylation signal.

MATERIALS AND METHODS

Plasmid Construction—Nucleic acid manipulations, such as restriction digests, nucleic acid 3′-end labeling, ligations, transformations, and DNA sequencing, were performed essentially as described (Maniatis et al., 1982).

The construction of the pSVB3/Ba plasmid and the generation of the unidirectional 3′ deletion mutants has been previously described (Woychik et al., 1984). Additional 3′ deletions were isolated from this library and characterized by chain termination DNA sequencing.

1The abbreviations used are: bGH, bovine growth hormone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid.
By subcloning portions of the bGH cDNA, the bGH introns were removed from the pSVB3/Ba expression vector to form pSVB3/BaΔIVS. A HinclI to Scal fragment of pSVB3/BaΔIVS containing the SV40 late promoter and intronless bGH gene was ligated into a HindIII- and HincII-digested pBS+ vector (Stratagene) to form pBS+bGHΔIVS. Two unidirectional deletions Δ+4, Δ+27, Δ+18, Δ+14, and Δ+10 were transferred from the pSVB3/Ba-based vector by digestion with SmaI and EcoRI and ligation into the EcoRI digested pBS+bGHΔIVSexp. The block and point mutations (m1-10, m16-17, m19-27, m alone, m19-24) were constructed by oligonucleotide-directed mutagenesis in a selectable vector as described (Setzer et al., 1990), confirmed by chain termination DNA sequencing, and subcloned into the pBS+bGHΔIVSexp plasmid. The internal control vector pBS+bGHΔIVSexp-SVL was formed by replacing the SmaI to EcoRI fragment containing the bGH polyadenylation signal in pBS+bGHΔIVSexp with a 186-nucleotide BamHI to DraI fragment of SV40 containing the SV40 late polyadenylation signal and 55 nucleotides of 3' flanking sequence (Fig. 1B).

The polymerase chain reaction was used to generate a 3' unidirectional deletion extending to immediately downstream of the hexanucleotide AATAAA to position Δ-19. The antisense oligonucleotide 5' -TCTATAGAAAAAGACA-3' was used to delimit the 3' end of the amplified region. The sense oligonucleotide 5' -CTCTCTCGTTCT-CTCCCTC-3' anneals to a region near the end of the last intron in the bGH gene in the vector pSVB3/Ba and generates a 310-nucleotide polymerase chain reaction product. The amplified DNA was digested with Xmal, and the resultant 262-nucleotide fragment was purified from an agarose gel. The Δ-19 deletion was inserted into the EcoRI site of pBS+bGHΔIVSexp by EcoRI digestion, removal of the 5' overhang with mungbean nuclease, secondary digestion with Xmal and ligation of the Xmal-digested polymerase chain reaction product into the pBS+bGHΔIVSexp vector.

Transfection and RNA Isolation—COS-1 cells were grown in 100-mm dishes and transfected when approximately 50% confluent as described (Camper et al., 1986). An internal control plasmid containing a 3'-truncated bGH gene fused to the SV40 late polyadenylation signal was cotransfected with another expression vector containing the bGH mutations of interest. Each transfection included 2 μg of the internal control vector pBS+bGHΔIVSexp-SVL and 4 μg of the assay plasmid in 3 μl of Dulbecco's modified Eagle's medium containing 50 mM HEPES (pH 7.2 at 37 °C) and 250 μg/ml DEAE-dextran (Sigma). The cell layers were washed twice with phosphate-buffered saline and once with Dulbecco's modified Eagle's medium. The transfection mix was then overlaid, and the cells were incubated for 2 h at 37 °C with gentle rocking. The transfection mix was removed, and the cell layers were again washed twice with phosphate-buffered saline and once with Dulbecco's modified Eagle's medium before fresh medium was added. The cells were allowed to grow another 2-3 days, and the cytoplasmic poly(A)+ RNA was isolated as described (Woychik et al., 1984).

Hybridization and S1 Nuclease Protection Assays—Double-stranded DNA probes were labeled with [α-32P]dCTP at their 3' ends with T4 DNA polymerase as described (Woychik et al., 1984). Twenty picomoles of probe and one-tenth of the poly(A)+ RNA from a plate of transfected COS-1 cells (approximately 100 ng) were ethanol-precipitated, dissolved in 10 μl of a hybridization mix containing 80% recrystallized formamide, 10 mM PIPES (pH 6.4), 400 mM NaCl, and 1 mM EDTA and denatured at a 10-μm incubation at 80 °C. The samples were allowed to hybridize for ~16 h at 53 °C before 30 μl of cold S1 nuclease buffer (30 mM sodium acetate (pH 4.6), 250 mM NaCl, 1 mM ZnSO4, and 20 μg/ml single-stranded salmon sperm DNA) containing 80 units of S1 nuclease (Boehringer Mannheim) was added, and the samples were incubated at 37 °C for 1 h. The reactions were then ethanol-precipitated, denatured, and resolved on a standard denaturing polyacrylamide gel. The gel was fixed and dried onto Whatman 3MM filter paper. The radioactive bands were visualized by autoradiography with Kodak X-OMAT AR film at ~70 °C. The same 3'-end-labeled DNA probe will hybridize to the transcripts from the wild-type bGH and internal control vector but will protect uniquely sized fragments following S1 nuclease digestion (Fig. 2A). A U. S. Biochemical Corp. Scican 5000 densitometer was used to quantitate these bands, and the ratio of the bGH polyadenylation site-derived signal to that of the SV40 late site was calculated. This construct protects the wild-type bGH and internal control vector but will protect the hybrids resulting from the construct containing the bGH polyadenylation signal and internal control transcripts corrected for any differences in the transfection efficiency, levels of expression, cytoplasmic poly(A)+ RNA recovery, probe-specific activity, hybridization, or loading of the electrophoresis gel. These "corrected" bGH values were then normalized to the value obtained from transfection with the wild-type bGH expression vector to simplify comparisons between the various mutants. To ensure that an excess of labeled transcripts were detected in the hybrids, a control was included in every experiment that contained half the mass of probe used in the remaining hybridizations. The equivalent signals obtained with the two levels of probe demonstrated that the hybridizations were conducted in probe excess. The ratios of the bGH to SV40 late polyadenylation signals and the effects of deletion relative to the wild-type bGH polyadenylation signal as calculated by densitometry were indistinguishable from the results obtained by scintillation counting of the excised bands from Fig. 2B.

RESULTS

Earlier work in our laboratory had indicated that the deletion of bGH gene 3'-flanking sequences to a position 84 nucleotides downstream (Δ+84) of the wild-type cleavage site did not significantly alter the efficiency or accuracy of polyadenylation. Further deletion to Δ+14 or Δ+10 resulted in the loss of accurate cleavage without a detectable decrease in efficient polyadenylation (Woychik et al., 1984). This estimation of efficiency relied on a simple transfection and nuclease protection assay that was unable to measure less than a 2-fold decrease in steady-state bGH mRNA levels. A more precise method involving cotransfection of the bGH mutations of interest with a chimeric bGH/SV40 late gene was developed to allow the dissection of efficiency elements by further analysis of deletions and point mutants in the 3'-flanking region of the bGH gene.

Efficiency Analysis of 3' Deletions—A previously described library (Woychik et al., 1984) of unidirectional deletions of the 3'-flanking sequences of the bGH gene was rescreened to identify clones whose deletion end points fell within the Δ+14 to Δ+84 region. Deletions were isolated that extended to Δ+69, Δ+53, Δ+44, Δ+27, and Δ+18 (Fig. 1A). We also used the polymerase chain reaction to generate a 3' deletion extending to 19 nucleotides upstream of the wild-type cleavage site (Δ-19). This deletion effectively removes all bGH sequences downstream of the hexanucleotide AATAAA while retaining the hexanucleotide and all upstream sequences. These bGH mutants were subcloned into the intronless bGH expression vector pBS+bGHΔIVSexp (Fig. 1B). Each deletion was cotransfected into COS-1 cells with the expression vector pBS+bGHΔIVSexp-SVL, which replaces the bGH polyadenylation signal with the SV40 late polyadenylation signal and the resultant poly(A)+ RNA analyzed by a quantitative S1 nuclease protection assay. The S1 nuclease strategy is diagrammed in Fig. 2A. The 3' end-labeled S1 probe will protect 674 nucleotides of the bGH transcript but loses homology with the internal control transcripts after 392 nucleotides. In Fig. 2B, a representative autoradiogram displays the resolution and low background obtainable with this method.

The deletion of bGH 3'-flanking sequences to Δ+84, Δ+69, or Δ+44 did not alter the efficiency of the bGH polyadenylation signal (Fig. 2, B and C). Further deletion to Δ+27 led to a modest decrease in polyadenylation efficiency to 65% that of the parental expression vector. Deletion to Δ+18 led to an additional drop in efficiency to 34% of wild type, also implicating the region between Δ+18 and Δ+27 in forming part of an efficiency signal. The deletions to Δ+14 and Δ+10 resulted in a further decrease in efficiency to 25% of wild-type levels. Thus, the entire region between Δ+10 and Δ+44 contains sequences important for the efficient polyadenylation of the bGH gene. This gradual decrease in polyadenylation efficiency with increasing deletion is in contrast with a
targeting the sequence UGU found at the core of a proposed consensus sequence of the polyadenylation efficiency element (McLauchan et al., 1985). A double mutant, m(19,24), was formed to disrupt a tandem repeat of the proposed CAYUG consensus element (Berget, 1984). As shown in Fig. 2D, each of the block mutations led to a 25–45% reduction in polyadenylation efficiency, corroborating the deletion analysis that identified each of these regions as part of an efficiency signal. However, none of the point mutations led to any alteration in the efficiency of polyadenylation. The modest decrease in efficiency exhibited by each of the block mutations and the failure of the point mutations to alter efficiency indicate that the bGH polyadenylation signal does not require a discrete GU-rich element.

Identification of an Accuracy Element—Earlier work has demonstrated that the deletion of bGH 3’-flanking sequences to Δ+84 retains >90% cleavage at the wild-type site, whereas further deletion to Δ+14 results in a pronounced shift in the polyadenylation cleavage site from the wild-type site to a variant cleavage site 5 nucleotides upstream (Woychik et al., 1984). This microheterogeneity in cleavage site choice is distinct from that involving a shift in the utilization of alternative polyadenylation signals as observed in the immunoglobulin heavy chain gene (Peterson et al., 1991). To more precisely delineate the accuracy element contained within this region of 3’-flanking DNA, the unidirectional, 3’ deletion mutants were assayed for accurate cleavage by transfection into COS-1 cells followed by S1 nuclease mapping using a radiolabeled probe short enough to differentiate between these two sites (Fig. 3). The deletion of bGH 3’-flanking sequence to Δ+27 did not alter wild-type site selection, but the deletion to Δ+18 led to partial use of the variant cleavage site. Further deletion to Δ+14 or Δ+10 shifted the preferred cleavage site to favor the variant site located 5 nucleotides upstream of the wild-type site. Because ~90% of the bGH transcripts derived from Δ+27 or the full-length 3’-flanking sequence were cleaved at the major site, whereas 75% utilized the variant site in Δ+14, the deletion of these nucleotides led to a 3.5-fold alteration in accuracy. Additional deletion to Δ+10 did not lead to any further loss of accuracy. These data suggest that an element responsible for the accurate cleavage of bGH pre-mRNA lies in the region between Δ+14 and Δ+27.

The block mutation m(19–27) caused a large loss in accuracy as deletion mutants Δ+14 or Δ+18, whereas the m(15–17) or m(10–14) mutations had little or no effect on accuracy. Thus the accuracy element seems to be bordered by the Δ+18 and Δ+27 deletions. The sequence within this region contains the repeated motif AUU, which is homologous to the sequence found at the wild-type cleavage site (see Fig. 1A). However, the m(19,24) mutation in which both adenosines of the AUU repeats are changed to cytosines had no effect on the accuracy of cleavage, indicating that the accuracy element, as well as the efficiency elements, were resistant to minor mutation.

We also examined the possible role of the AUU repeat as a consensused accuracy element by subjecting a variety of polyadenylation signals to statistical analysis. Using the binomial distribution to analyze the region between Δ+17 and Δ+30 in 102 polyadenylation signals, we found no statistically significant occurrence of the sequence AUU (data not shown).

**DISCUSSION**

The bGH polyadenylation signal exhibits sequence requirements that distinguish it from other, well characterized mammalian polyadenylation signals. Unidirectional, 3’ deletions display a gradual decrease in polyadenylation efficiency, cul-
minating in a 4-fold reduction with deletion to $\Delta+10$. Block mutations within the region $\Delta+10$ to $\Delta+27$ each lead to a modest 25–45% decrease in efficiency, whereas point mutations have no effect. We have observed no dependence of polyadenylation on GU- or U-rich elements. In contrast, block mutagenesis of the GU element in the herpes simplex virus thymidine kinase (Zhang et al., 1986) or the rabbit $\beta$-globin (Gil and Proudfoot, 1987) polyadenylation signals leads to a greater than 4-fold decrease in efficiency, and point mutants of the SV40 early GU element decreases efficiency 5-fold (McDevitt et al., 1986). Our results are not consistent with the model of a discrete efficiency element in the 3'-flanking region such as is found in the SV40 early, SV40 late, herpes simplex virus thymidine kinase, adenovirus E2A, and rabbit $\beta$-globin polyadenylation signals.

Several motifs have been proposed for polyadenylation elements downstream of the conserved AAUAAA sequence. The herpes simplex virus thymidine kinase (Zhang et al., 1986) or rabbit $\beta$-globin (Gil and Proudfoot, 1987) polyadenylation signals display a high sensitivity to small deletions or block mutations in specific GU- or U-rich sequences. Conversely, the SV40 early and adenovirus E2A polyadenylation signals are very resistant to minor internal deletions or block mutations because these signals contain functionally redundant downstream efficiency elements. The prior disruption of one of these redundant elements is necessary to unmask the effects of block or point mutations in the second element (McDevitt et al., 1986). The SV40 late polyadenylation signal also displays a high resistance to methylation interference (Conway and Wickens, 1987) and linker scanner mutations (Zarkower and Wickens, 1988) and has been found to contain redundant efficiency elements (Qian and Wilusz, 1991).

Both of these paradigms are distinct from results obtained with the bGH polyadenylation signal. We do not identify any downstream sequences, such as GU or U motifs, whose disruption leads to a dramatic loss of efficiency. Nor do we from the bGH transcripts and those from the bGH/SV40 late fusion transcripts are shown, as well as a small amount of reannealed probe protected from S1 nuclease digestion. In the probe alone lane, no COS-1 poly(A$^+$) RNA is included in the hybridization. The $pB$Sexp lane is from a hybridization of A$^+$ RNA from bGH/SV40 late transfectected COS-1 cells containing one-half the mass of probe used in the other hybridization signals and is included as a control demonstrating that the hybridizations were conducted in probe excess. The other lanes contain hybridizations of the indicated unidirectional 3' deletions, C and D, combined data from independent transfections for the 3' deletions and the mutations, respectively. The error bars represent 1 S.E. of the mean for the combined data sets.
identify redundant efficiency elements that would mask the effects of individual block mutations. Rather, we observe a gradual loss of efficiency with increasing deletion and a small, but highly reproducible, loss of efficiency in three separate block mutations spanning the region Δ+10 to Δ+27. This observation is also distinct from that in the Xenopus β-globin polyadenylation signal where there appear to be no efficiency elements in the 3'-flanking region (Mason et al., 1986).

The observed lack of a requirement for discrete GU- or U-rich sequences might be expected if the bGH polyadenylation signal were inefficient, as compared with other signals. However, the bGH polyadenylation signal has been shown to be efficient in the R1610 hamster cell line (Pfarr et al., 1986) and has an efficiency comparable with that of the SV40 early and herpes simplex virus thymidine kinase gene polyadenylation signals in transfected COS-1 cells.3 These observations are not unique to COS-1 cells because the changes in efficiency and accuracy of the bGH deletions and mutations relative to the wild-type bGH gene are identical in transfected CHOB52 cells (data not shown). As the deletion and block mutations independently identify the same sequences as part of an efficiency signal, it is also unlikely that our observations are the result of interference by nonspecific sequences introduced during construction of the mutations.

Though much effort has been focused on defining the sequence elements necessary for polyadenylation efficiency, little is known about the sequence requirements for the accurate positioning of the cleavage site. A general preference for cleavage at CA dinucleotides 10–30 nucleotides downstream of the hexanucleotide has been observed (McLauchlan et al., 1985). However, these distance and sequence constraints are insufficient to explain cleavage site preference, because many polyadenylation signals contain sites that appear compatible for cleavage but are not utilized. We have identified a discrete accuracy element in the bGH polyadenylation signal located between Δ+18 and Δ+27. The unidirectional 3' deletion to Δ+18 and the m(19–27) block mutation both disrupt this element, promoting the use of a cryptic cleavage site 5 nucleotides upstream of the wild-type site. We also note that although the accuracy and efficiency elements overlap in m(19–27), there is no requisite association between such elements because both the m(10–14) and m(15–17) mutations decrease the efficiency without altering the accuracy of polyadenylation. Deleting portions of the Xenopus β-globin 3'-flanking sequence also led to alterations in accuracy (Mason et al., 1986), suggesting that such accuracy elements may be a common feature in a number of nonviral polyadenylation signals.

Given the rapid progress in understanding the biochemistry of polyadenylation, it may soon be possible to probe the unusual sequence requirements of the bGH polyadenylation signal for accurate and efficient polyadenylation and gain an understanding of the mechanisms that allow this signal to retain high efficiency in the absence of GU- or U-rich sequences.

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REFERENCES


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