MOLECULAR AND CELLULAR BIOLOGY, Apr. 1989, p. 1604–1610 Vol. 9, No. 4
0270-7306/89/041604-07$02.00/0
Copyright © 1989, American Society for Microbiology

Alternative Processing of Bovine Growth Hormone mRNA Is Influenced by Downstream Exon Sequences

ROBERT K. HAMPSON,* LIZELLEN LA FOLLETTE, AND FRITZ M. ROTTMAN

Department of Molecular Biology and Microbiology, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

Received 23 September 1988/Accepted 10 January 1989

In a previous report, we described the presence, in pituitary tissue, of an alternatively processed species of bovine growth hormone mRNA from which the last intron (intron D) has not been removed by splicing (R. K. Hampson and F. M. Rottman, Proc. Natl. Acad. Sci. USA 84:2673–2677, 1987). Using transient expression of the bovine growth hormone gene in Cos I cells, we observed that splicing of intron D was affected by sequences within the downstream exon (exon 5). Deletion of a 115-base-pair *Fsp*I-*Pvu*II restriction fragment in exon 5 beginning 73 base pairs downstream of the intron 4-exon 5 junction resulted in cytoplasmic bovine growth hormone mRNA, more than 95% of which retained intron D. This contrasted with less than 5% of the growth hormone mRNA retaining intron D observed with expression of the unaltered gene. Insertion of a 10-base-pair inverted repeat sequence, CTTCCGGAAG, which was located in the middle of this deleted segment, partially reversed this pattern, resulting in cytosolic mRNA from which intron D was predominantly removed. More detailed deletion analysis of this region indicated that multiple sequence elements within the exon 5, in addition to the 10-base-pair inverted repeat sequence, are capable of influencing splicing of intron D. The effect of these exon sequences on splicing of bovine growth hormone precursor mRNA appeared to be specific for the growth hormone intron D. Deletions in exon 5 which resulted in marked alterations in splicing of growth hormone intron D had no effect on splicing when exon 5 of bovine growth hormone was placed downstream of the heterologous bovine prolactin intron D. The results of this study suggest a unique interaction between sequences located near the center of exon 5 and splicing of the adjacent intron D.

The structural region of the gene that encodes bovine growth hormone spans approximately 1.8 kilobases and consists of five exons interrupted by four introns (34). The mature, fully spliced mRNA is 814 nucleotides long (excluding polyadenylation); the sequence contains 58 and 105 nucleotides of 5′ and 3′ untranslated regions, respectively. We reported previously the presence in bovine pituitary of an alternatively processed species of bovine growth hormone mRNA from which the last intervening sequence (intron D) is not removed by splicing (13). The retained intron D includes an additional 274 nucleotides in the mRNA, with maintenance of an open reading frame across the intron. A termination codon is encountered 50 nucleotides into exon 5, which is shifted from the normal reading frame in this alternatively processed mRNA. Subsequent translation would result in a growth hormone-related polypeptide with 125 amino-terminal amino acids identical to those of wild-type growth hormone, followed by an alternate carboxyl terminus of 108 amino acids. This alternate form of growth hormone would be 42 amino acids longer than wild-type bovine growth hormone, or approximately 5,000 greater in molecular weight. We demonstrated that intron D-containing bovine growth hormone mRNA is found on polysomes, which suggested that this mRNA species is translated into a polypeptide. Subsequently, identical alternative processing (i.e., retention of intron 4) was reported with mRNA of the human growth hormone variant gene (6); these findings also predicted a similar high-molecular-weight variant species of human growth hormone. Of the four introns of the bovine and human growth hormone genes, intron D is unique in that there exists a considerable degree of similarity between the respective intron D sequences of the two genes (12).

In an investigation initiated to examine the polyadenylation signal of bovine growth hormone pre-mRNA via progressive deletion of the region around the polyadenylation site (E. C. Goodwin and F. M. Rottman, J. Cell. Biochem. **10**(Suppl.)**D**:170, 1986), it was observed that several constructs containing deletions in exon 5 resulted in cytosolic mRNA of larger than expected size. We then determined that this increase in size resulted from retention of intron D. With transient expression of the normal, wild-type gene in Cos I cells, intron D is retained in approximately 2% of the total cytosolic mRNA, similar to observations with stably transfected Chinese hamster ovary cells (13). Exon 5 of the bovine growth hormone gene is 303 nucleotides long. Deletions in exon 5 beginning at a *Sma*I site 23 nucleotides downstream of the splice acceptor site and ranging from approximately 150 to 246 base pairs (bp) dramatically altered splicing such that greater than 95% of the polysomal growth hormone mRNA retained intron D. The objective of the investigation presented here was to more closely examine the role of sequences within exon 5 of the bovine growth hormone precursor mRNA in influencing the efficiency of splicing of intron D and to determine the degree to which this observed influence of sequences within exon 5 on splicing also is dependent on sequences with intron D.

MATERIALS AND METHODS

Construction of bovine growth hormone deletion clones. The parent expression plasmid, pSVB3/Ba (35), contains the entire structural region of the bovine growth hormone gene plus 438 bp of 3′-flanking sequence driven by the simian virus 40 late-region promoter. All deletions in exon 5 described herein are denoted by a Δ followed by an indication

* Corresponding author.

of the region deleted (see Fig. 1A and 2A). Region A extends from a *Sma*I site at position 22 to an *Fsp*I site at position 73, region B extends from the *Fsp*I site to a *Pvu*II site at position 188, and region C extends from the *Pvu*II site to a deletion point 34 nucleotides from the polyadenylation site (see below). The plasmid containing the large deletion of exon 5 (ΔABC) was constructed by nuclease BAL 31 digestion from the *Sma*I site 22 bp downstream of the intron-exon junction in exon 5 to within 34 nucleotides of the polyadenylation site (Goodwin and Rottman, J. Cell. Biochem. **10**(Suppl.)**D**:170, 1986). This plasmid also contains a major deletion of the 3'-flanking region to within 18 nucleotides of the polyadenylation site. All other deletions were constructed by standard methods (20), using convenient restriction sites. The plasmid containing deletion of region C (ΔC) was constructed by insertion of the *Sma*I-*Pvu*II fragment (i.e., regions A and B) into the clone containing the large deletion (ΔABC) by virtue of *Sma*I linkers used to construct this plasmid. As a result, there is a 4-bp remnant of the *Sma*I linkers, GGGC, located at the site of this deletion.

Selective deletion and insertion of the inverted repeat sequence CTTCCGGAAG. The 10-nucleotide inverted repeat sequence CTTCCGGAAG was deleted specifically by using oligonucleotide-directed mutagenesis as described by Zoller and Smith (36) except that bacteriophage T4 polymerase was used instead of the Klenow fragment of DNA polymerase I. The sequence of the region around the inverted repeat and the resulting sequence following this deletion are illustrated in Fig. 3. This resulting sequence, which resembled the original inverted repeat, was deleted in an identical manner, along with an extra 2 nucleotides, such that no other inverted repeat sequences resulted. Insertion of the inverted repeat CTTCCGGAAG was accomplished by the linker tailing technique (16). These clones are represented schematically in Fig. 4.

Construction of bovine growth hormone-prolactin chimeric genes. The growth hormone exon 5-prolactin intron D chimeric genes were constructed from a prolactin minigene construct (provided by S. M. Carroll) containing exons 1 through 4, intron D, and exon 5. The prolactin gene was truncated at a *Ban*II site which, after conversion to a blunt end with T4 polymerase (20), left the first 25 bp of prolactin exon 5. Exon 5 of growth hormone was ligated to this truncated prolactin gene by using a *Sma*I site located 22 nucleotides downstream of the intron D-exon 5 junction.

DNA transfection of Cos I cells. Cos I cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and antibiotics (penicillin and streptomycin; GIBCO Laboratories, Grand Island, N.Y.) and transfected by a modified DEAE-dextran procedure as described previously (4). Cells were harvested, and polysomal RNA was prepared 48 h after transfection.

Preparation of polysomal mRNA. Polysomal mRNA was prepared by a modification of a procedure described elsewhere (23). The translation elongation inhibitor emetine was added to the medium at a final concentration of 100 μg/ml for 10 min before harvesting of the cells to prevent ribosomal runoff during the isolation procedure. Briefly, the cells were washed three times with phosphate-buffered saline and detached via a 10-min incubation with 2 mM EDTA in phosphate-buffered saline. The cells were sedimented, and the pellet was lysed at room temperature in 1 ml of 10 mM Tris chloride (pH 7.4), 10 mM NaCl, 1 mM $MgCl_2$, 0.5% Nonidet P-40, and 10 mM vanadyl ribonucleoside complexes (2). After chilling on ice, the nuclei and other particulate material were sedimented at 8,000 × *g* for 10 min; the resulting supernatant was layered on a cushion (4 ml) of 30% sucrose in the lysis buffer (minus vanadyl complexes and Nonidet P-40). The polysomes were sedimented at 45,000 rpm for 2 h at 4°C in a 50.3Ti rotor, using heat seal tubes. Polyadenylated RNA was prepared by oligo(dT)-cellulose chromatography (20).

Nuclease S1 mapping of RNA. Probes for nuclease S1 protection experiments were 3' end labeled with T4 DNA polymerase and [α-^{32}P]dCTP. The vector was digested briefly with the 3'-exonuclease activity of T4 polymerase before addition of nucleotides (20). The probes used for mapping bovine growth hormone and prolactin are indicated in Fig. 1 and 5, respectively. Approximately 5 ng of probe was coprecipitated with 10% of the poly(A)$^+$ RNA isolated from a single 10-cm-diameter dish of transfected cells. The samples were redissolved in 80% formamide, 0.4 M NaCl, 10 mM piperazine-*N*,*N'*-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), and 1 mM EDTA, heated to 75°C for 10 min, and then transferred to 57.5 and 53°C for the growth hormone and prolactin probes, respectively. After hybridization overnight, the samples were digested at 42°C with 80 Vogt units of S1 nuclease (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in 90 μl of 0.25 M NaCl, 0.03 M sodium acetate (pH 4.6), and 1.0 mM $ZnSO_4$. After incubation for 1 h, the samples were precipitated with 200 μl of ethanol and analyzed on a 6% polyacrylamide–urea sequencing gel.

RESULTS

Deletion of exon 5 sequences alter efficiency of splicing of intron D. As a first step to delineate the region(s) within exon 5 that might be involved in this altered splicing of intron D, convenient restriction sites were used to introduce various deletions into exon 5, which for ease of discussion will be referred to as regions A, B, and C (Fig. 1A). The original plasmid containing the large deletion of exon 5 (ΔABC), extending from bases 23 through 269 out of a total exon length of 303 bases, also contains a large deletion of the 3'-flanking region to within 18 nucleotides of the polyadenylation site. Deletion of the 3'-flanking region was found to have no effect on splicing of intron D (data not shown). These constructs were expressed transiently in Cos I cells, and the resulting polysomal poly(A)$^+$ mRNA was analyzed for splicing of intron D by S1 nuclease protection mapping (Fig. 1B). The results of these experiments (Fig. 1C) indicated that deletion of region B (ΔB) influenced splicing of intron D identically to that observed with the large deletion (ΔABC); i.e., there was greater than 95% retention of intron D. In contrast, deletion of region A (ΔA) or region C (ΔC) did not result in any increase in retention of intron D. In fact, deletion of region A consistently resulted in a slight increase in the efficiency of removal of intron D. Simultaneous deletion of regions A and C (ΔAC) also did not result in increased retention of intron D (Fig. 2, clone ΔAC).

The responsible sequence is position independent and is effective in both orientations. To further demonstrate a positive influence of sequence(s) within region B on the efficiency of intron D splicing, the region B fragment was reinserted into the clones from which this sequence had been deleted (ΔABC and ΔB) (Fig. 2A). As would be expected if a sequence(s) within region B were necessary for efficient splicing of intron D, ligation of the *Fsp*I-*Pvu*II fragment into clones from which region B had been deleted restored the efficiency of intron D splicing to that observed with the wild-type exon 5 (Fig. 2, clones ΔAC and BAC). Unexpectedly, insertion of the *Fsp*I-*Pvu*II fragment into the *Sma*I site

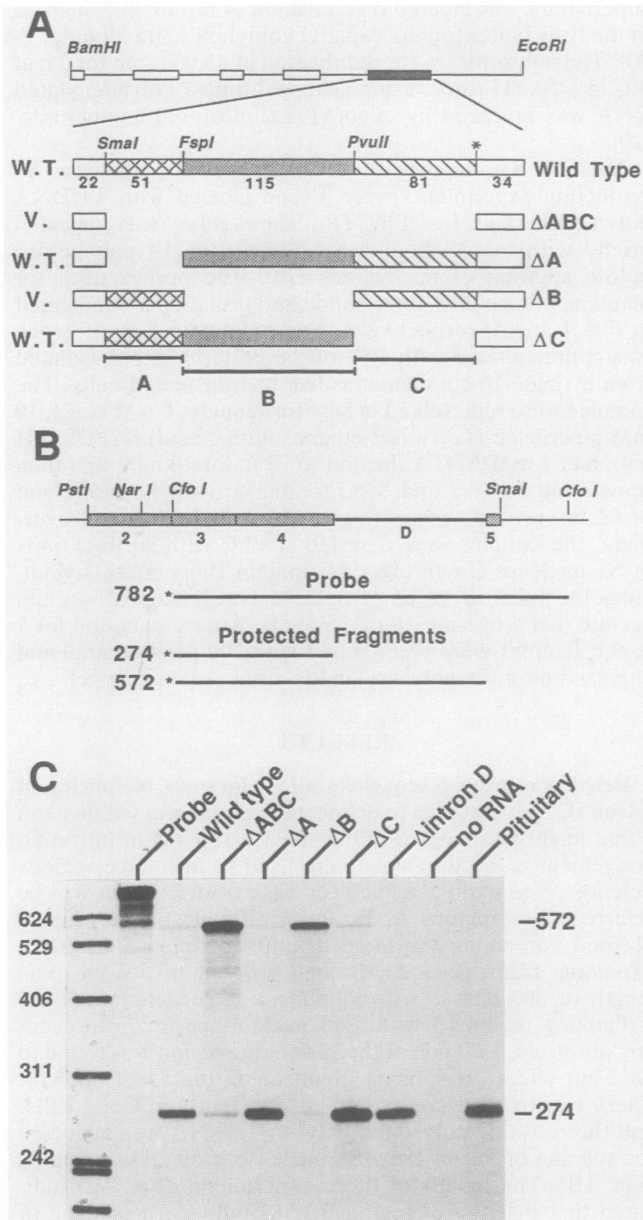

FIG. 1. Deletion of an internal region within exon 5 of the bovine growth hormone gene, resulting in retention of intron D in polysomal mRNA transiently expressed in Cos I cells. (A) Schematic representations of the bovine growth hormone gene and deletions introduced into exon 5 by using convenient restriction sites. Restriction sites used are as indicated; regions between the sites are designated A, B, and C. *, Endpoint of a nuclease BAL 31 deletion used in construction of clones ΔABC and ΔC (see Materials and Methods). Removal of intron D from these constructs is summarized at the left (W.T., wild type, or greater than 95% removal of intron D; V., variant or alternatively processed, indicating greater than 95% retention of intron D). (B) Nuclease S1 mapping strategy to determine splicing or retention of intron D. The probe was a *Cfo*I-*Cfo*I fragment 3' end labeled with T4 DNA polymerase and [α-^{32}P]dCTP (see Materials and Methods). The shaded boxes, heavy line, and thin line represent exon, intron, and vector sequences, respectively. (C) Nuclease S1 mapping of polysomal mRNA isolated from Cos I cells transiently expressing the constructs depicted in panel A. Species of mRNA corresponding to fully spliced and intron D-containing mRNA are indicated by the bands at 274 and 572 nucleotides, respectively. Size markers at the left (in base pairs) are pBR322 restricted with *Hpa*II and labeled with [α-^{32}P]dCTP and Klenow fragment of DNA polymerase I.

of these clones in orientation reverse of that in the wild-type exon 5 also restored the efficiency of splicing of intron D to that observed with the wild-type exon (Fig. 2, clones ΔAC/invB and invBAC). Unlike the case with typical promoter enhancers, which retain the original sequence while functioning in either orientation, reversal of a sequence transcribed into RNA results in a completely new sequence. This result might argue a possible position or exon size requirement for efficient splicing. However, we observed no correlation between exon length and splicing. The total length of exon 5 containing the deletion of region B (Fig. 1, ΔB) was 188 bases and intron D was not spliced, whereas the clones in which region B was inserted into the large deletion had shorter exon lengths of 176 nucleotides (Fig. 2, ΔAC and ΔAC/invB), and intron D was spliced with the same efficiency as was the wild-type gene. An alternative explanation for this observation would be the presence of a sequence within region B which would be transcribed in either orien-

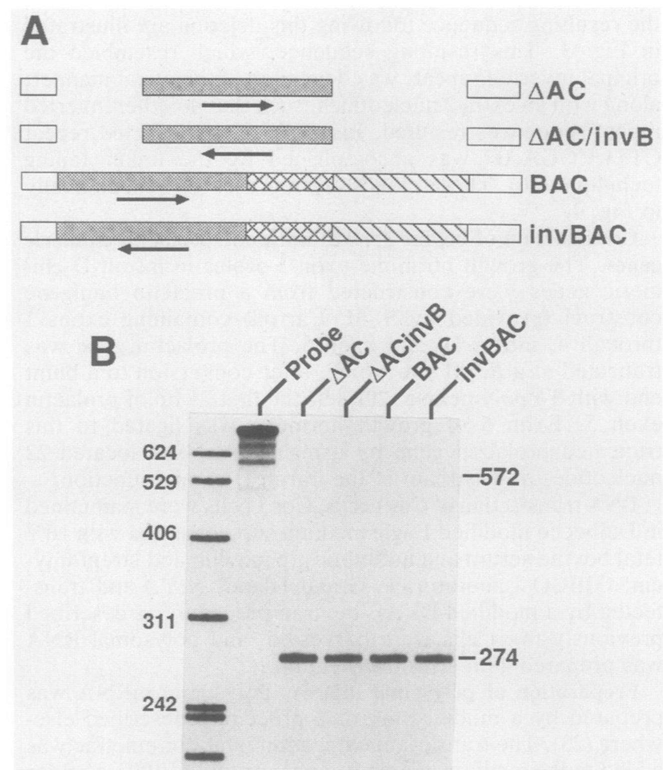

FIG. 2. Restoration of splicing of intron D by insertion of the 115-bp *Fsp*I-to-*Pvu*II fragment (region B) into the *Sma*I site of the ΔABC and ΔB deletion clones. (A) Schematic representations of constructs containing insertion of the region B fragment. Regions and shading are as described in the legend to Fig. 1; arrows indicate orientation of the region B fragment relative to normal orientation in the gene. (B) Nuclease S1 mapping of polysomal mRNA isolated from Cos I cells transiently transfected with the constructs depicted in panel A. The autoradiogram is from the same experiment, with identical exposure time, as that shown in Fig. 1. Size markers on the left are indicated in base pairs.

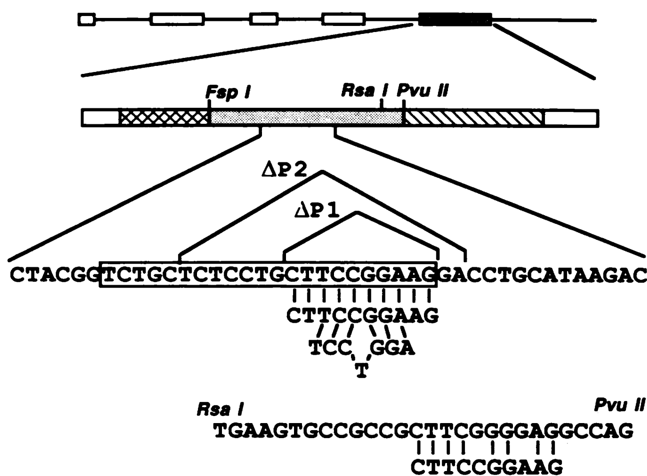

FIG. 3. Schematic representation of the bovine growth hormone gene indicating various sequence features within region B of exon 5. Locations of the 10-bp inverted repeat sequence and the similar sequence at the 3′ end of region B (region B3′) are indicated. Deletion of the 10-base inverted repeat sequence (ΔP1) and subsequent deletion of the resulting 7-bp sequence (ΔP2) resembling the central portion of the original sequence were done by oligonucleotide-directed mutagenesis. The resulting 7-bp sequence resembling the core of the 10-bp inverted repeat sequence is indicated below. The box indicates the sequence similar to that of a splice acceptor within this region (see Discussion).

tation. Analysis of this region revealed the presence of a 10-base inverted repeat, CTTCCGGAAG, beginning 44 nucleotides downstream of the center of the *Fsp*I site (Fig. 3).

Effects of exon 5 sequences on splicing of intron D involves multiple sequence elements. To test the hypothesis that the inverted repeat, CTTCCGGAAG, exerted a positive influence on splicing of intron D, the sequence was synthesized as a 10-bp linker and placed within exon 5 of the clone from which region B had been deleted (Fig. 4A, clone ΔB/Pal). Analysis of the resulting mRNA indicated that insertion of these 10 bp reversed the efficiency of intron D removal such that intron D was now predominantly spliced.

In the parallel experiment, site-directed mutagenesis was used to specifically delete the 10-bp inverted repeat (Fig. 4, clone ΔP1). The result of this experiment indicated only a modest increase in intron D retention. However, upon further examination of the sequence of region B, we noted that deletion of the 10-bp inverted repeat resulted in the new sequence, TCCTGGA, which resembled the core sequence of the inverted repeat CTTCCGGAAG (Fig. 3). In addition, there was a sequence located near the 3′ end of region B, CTTCGGGGAG, which matched 8 of 10 bp of the inverted repeat (Fig. 3). To examine the potential involvement of these homologous sequences, clones containing deletions of these sequences also were constructed, the former by site-directed mutagenesis and the latter by deletion of an *Rsa*I-*Pvu*II restriction fragment. The retention of intron D in cytosolic mRNA upon expression of these clones (Fig. 4) is consistent with the hypothesis that these homologous sequences also play a role in influencing the splicing of intron D. Deletion of the region at the 3′ end of region B that contained the sequence 80% homologous with the inverted repeat significantly altered intron retention such that approximately 50% of polysomal mRNA possessed unspliced intron D (Fig. 4, clone ΔB3′). When this deletion of region B3′ was made in conjunction with deletion of the inverted repeat and

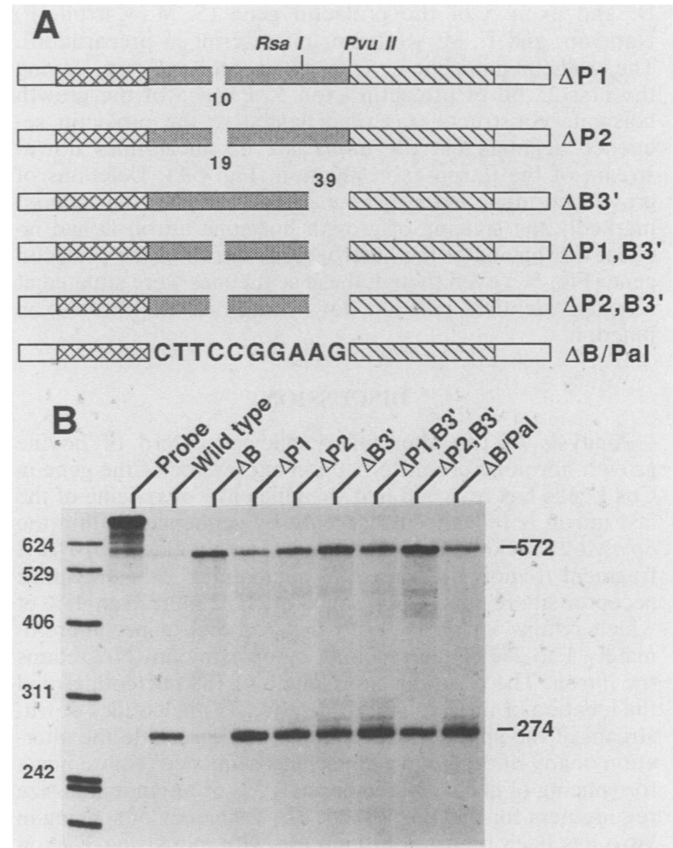

FIG. 4. Insertion and deletion of the 10-bp inverted repeat and sequences homologous to the inverted repeat, indicating that multiple sequence elements within region B of exon 5 are capable of influencing splicing of bovine growth hormone intron D. (A) Schematic representations of constructs containing the 10-base inverted repeat sequence inserted into the region B deletion and various deletions within region B, including the inverted repeat sequence and related sequences (see text). (B) Nuclease S1 mapping of polysomal mRNA isolated from Cos I cells transiently transfected with the constructs depicted in panel A. All conditions and the S1 mapping strategy are as described in the legend to Fig. 1. Size markers shown on the left are indicated in base pairs.

the sequence resembling the core of the inverted repeat (Fig. 4, clone ΔP2,B3′), retention of intron D in polysomal mRNA approached that observed with deletion of the entire region B. The results of these experiments suggest that the observed influence of exon 5 sequences on splicing of intron D is more complex than the simple presence or absence of a discrete sequence such as the 10-bp inverted repeat but likely involves multiple sequences within region B that are capable of influencing splicing of intron D.

Influence of bovine growth hormone exon 5 sequences on splicing are specific for the bovine growth hormone intron D. To determine whether sequences within exon 5 that influence splicing of intron D interact specifically with the adjacent intron D of the bovine growth hormone precursor mRNA or whether this exon might influence splicing of heterologous introns, several chimeric genes were constructed. Deletions in exon 5 which markedly increased retention of intron D in bovine growth hormone mRNA were placed downstream of intron D of the bovine prolactin gene. These chimeric constructions were made by using a prolactin minigene construct containing exons 1 through 4, intron

D, and exon 5 of the prolactin gene (S. M. Carroll, P. Narayan, and F. M. Rottman, manuscript in preparation). The prolactin minigene was truncated at a *Ban*II site, leaving the first 25 bp of prolactin exon 5. Exon 5 of the growth hormone constructs was then ligated to the prolactin sequence beginning at the *Sma*I site 22 nucleotides downstream of the intron-exon junction (Fig. 5A). Deletions of growth hormone exon 5 (i.e., ΔABC and ΔB) that altered markedly the splicing of growth hormone intron D had no effect on splicing of the heterologous intron of the prolactin gene (Fig. 5C) even though these sequences were situated at a nearly identical position downstream of the intron-exon junction.

DISCUSSION

Analysis of the alternative splicing pattern of bovine growth hormone pre-mRNA upon expression of the gene in Cos I cells has revealed that the efficiency of splicing of the last intron is influenced markedly by sequences within the downstream exon. Deletion of a 115-nucleotide *Fsp*I-*Pvu*II fragment (region B) located 73 nucleotides 3′ of the splice acceptor site results in polysomal mRNA, more than 95% of which retains intron D. With the unaltered gene, approximately 1 to 2% of the resulting cytoplasmic mRNA retains the intron. The resulting exon length of 188 nucleotides and the location of the *Fsp*I-*Pvu*II deletion 73 nucleotides downstream of the splice acceptor site would preclude the alteration of any of the known consensus sequence requirements for splicing of eucaryotic introns (1, 33) or any minimal size requirement for the 3′ exon (25, 27). Efficiency of splicing in vitro has been reported to increase with increasing 3′ exon length but reaches a plateau at approximately 100 nucleotides (10). Moreover, we observed no correlation between exon size and intron retention in these studies.

A striking aspect of this study was the observation that the pattern of intron retention upon deletion of the *Fsp*I-*Pvu*II fragment (region B) was substantially reversed by insertion of only a 10-bp inverted repeat (CTTCCGGAAG) from the middle of this region. The mechanism by which this sequence or other, similar sequences positively influence splicing while located at a considerable distance from the splice acceptor site is not immediately obvious. Mutational analysis of these 10 bp will provide a means to assess the essential features of this sequence in influencing splicing of intron D. The results of these analyses will likely be complex, as further deletion analyses of the *Fsp*I-*Pvu*II region suggested that multiple sequence elements within this region are capable of influencing splicing efficiency. Therefore, it will be necessary to determine not only the essential features of the inverted repeat sequence or similar sequences but also the number and relative importance of the sequences. The effects of these sequences on splicing are largely position independent. However, the observation that deletion of region A (see also Fig. 2, clones ΔAC, ΔAC/invB, BAC, and invBAC) results in an increase in efficiency of intron D removal suggests that the proximity to intron D of important sequences also may be of importance.

One possible mechanism by which the inverted repeat sequence or similar sequences influences splicing efficiency may be by providing a binding site for one or more *trans*-acting factors which mediate access to or binding of splicing components to intron sequences. Evidence for the involvement of specific *trans*-acting factors in the regulation of alternative processing includes genes encoding calcitonin (7, 18), the μ chain of immunoglobulin M (17), and troponin T

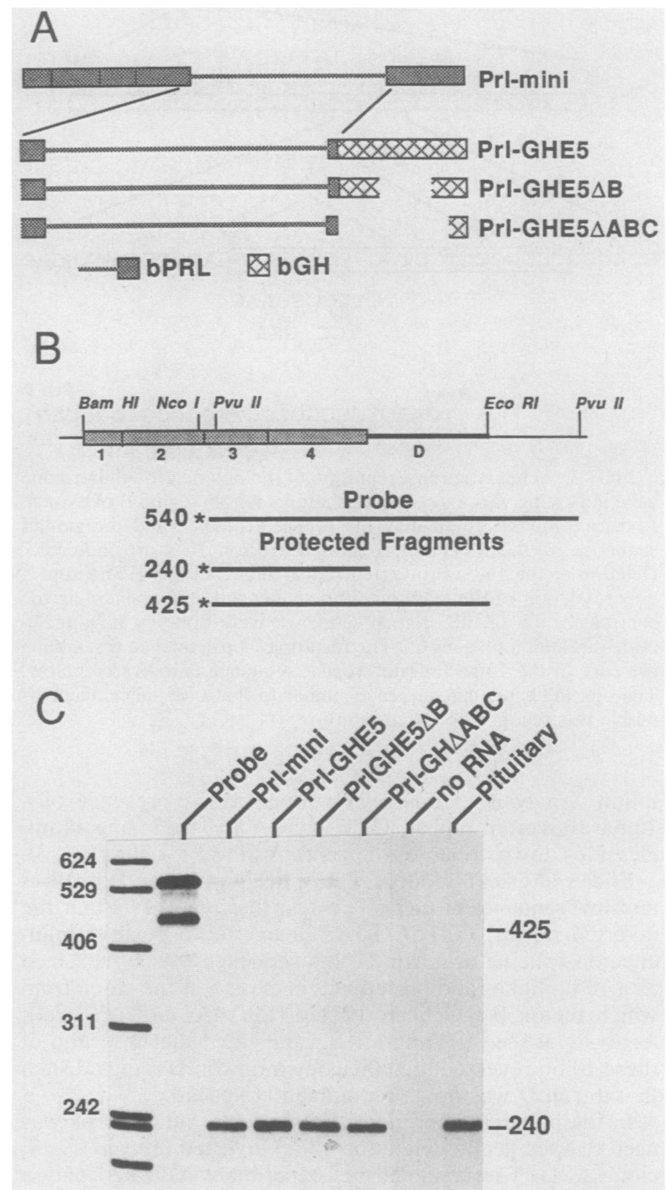

FIG. 5. Evidence that deletions of sequences within exon 5 of bovine growth hormone do not alter splicing when located downstream of the heterologous prolactin intron D. (A) Schematic representations of the growth hormone (bGH)-prolactin (bPRL) chimeric genes. The prolactin portion of the genes is derived from a minigene construct that lacks introns A, B, and C but contains intron D. The clones retain the first 25 nucleotides of prolactin exon 5 and begin at nucleotide 23 of growth hormone exon 5. (B) Nuclease S1 mapping strategy for determining efficiency of splicing of the prolactin intron D. The probe consisted of a *Pvu*II-*Pvu*II fragment 3′ end labeled identically to the probe used to analyze splicing of the growth hormone intron D. The boxes, heavy line, and thin line represent exon, intron, and vector sequences, respectively. (C) Nuclease S1 mapping of polysomal mRNA isolated from Cos I cells transiently expressing the growth hormone-prolactin chimeric gene constructs depicted in panel B. Species of mRNA corresponding to fully spliced and intron D-containing mRNA are indicated by the bands at 240 and 425 nucleotides, respectively. The expected position of the band corresponding to the intron D-containing form, along with hybridization conditions (see Materials and Methods), was verified by using in vitro-synthesized transcripts (data not shown). Size markers shown on the left are indicated in base pairs.

(3). This hypothesis is supported by the fact that the steady-state level of mRNA resulting from expression of clones yielding primarily unspliced intron D was not measurably less than that observed upon expression of the wild-type gene even though the mRNA possessed an intact and presumably functional intron. Ordinarily, mRNAs containing introns are not transported to the cytosol, ostensibly because binding of the small nuclear ribonucleoproteins or other factors in formation of the splicing complex commits the intron to removal (28). This implies that the splice donor and acceptor sites of intron D are not behaving independently and that in the absence of appropriate sequences within the exon, formation of the splicing complex does not occur. It may be that sequences within exon 5 of bovine growth hormone pre-RNA, through a *trans*-acting factor, serve to mediate the access of splicing components to their respective binding sites within the intron. These might include U1 or U2 small nuclear ribonucleoproteins, which are involved in the early steps of splicing complex formation (for reviews, see references 21 and 28), other protein factors that bind to regions within the intron (11, 31), or perhaps a factor similar to the splice commitment factor described in yeast cells (19).

Alternatively, this sequence might influence splicing complex formation via RNA intramolecular secondary-structure interactions. It has been demonstrated in vitro that alternative mRNA processing can be influenced by synthetically introduced RNA secondary structure (29), although this secondary structure had little effect on processing in vivo. A computer-assisted RNA folding algorithm (37) does not reveal any prominent interactions of sequences within exon 5 of bovine growth hormone mRNA with other regions of the molecule or any striking changes in the predicted secondary structure of intron D upon deletion of the region B or insertion of the 10-bp inverted repeat. However, this finding is not definitive evidence that important structures do not exist. Clearly, RNA secondary structure can be important for splicing, as evidenced by the group I and group II introns (for review, see reference 5); hence, the involvement of RNA secondary structure in alternative mRNA processing cannot be summarily dismissed. The sequence CUUCGG is often associated with particularly stable RNA stem-loop structures (32), and this sequence bears a noticeable resemblance to the initial portion of the 10-nucleotide inverted repeat (CUUCCGG) which substantially reversed the splicing pattern of the *Fsp*I-*Pvu*II deletion. Moreover, the *Rsa*I-*Pvu*II fragment, deletion of which results in retention of intron D in approximately 50% of cytosolic mRNA, contains a perfect copy of this sequence. In bovine growth hormone mRNA, however, there is no evidence suggesting that these sequences are associated with stem-loop structures, and whether they can be involved in stabilization of secondary structure remains to be determined.

A similar effect of exon sequences on alternative processing, in which a deletion of an internal 81 bp out of 270 bp of the EDIIIA alternatively spliced exon of fibronectin mRNA results in 100% exclusion of the exon in the mature mRNA, has been reported (22). There also is a report demonstrating that mutation of exon sequences alters the relative ratios of splice site usage in processing of the simian virus 40 late transcripts (30). There is an example in which exon sequences influence removal of a single specific intron. Insertion of a sequence resembling the polypyrimidine tract and branch point sequences into exon 2 of the β-globin gene poisoned splicing in in vitro extracts (10). It is worth noting that the *Fsp*I-*Pvu*II fragment (region B) contains a sequence that is very similar to a splice acceptor site, possessing considerable similarity to the natural splice acceptor site located at the end of intron D (Fig. 3). However, it would be difficult to explain the results with alternative processing of bovine growth hormone mRNA by a poison hypothesis. Inhibition of splicing was observed after deletion rather than insertion of sequences, including one that resembled a splice acceptor, and no obvious common sequence was created by the deletions that resulted in retention of intron D.

The apparent specificity of the sequences within exon 5 of growth hormone to influence removal of only the growth hormone intron and not the heterologous prolactin intron D suggests strongly that sequences within the growth hormone intron also are important in retention of this intron. There is precedence for the importance of intron sequences in controlling alternative processing. A "relatively poor" 3′ splice acceptor site appears to be involved in determining the steady-state level of spliced versus unspliced NS1 (nonstructural protein) mRNA of influenza virus (26). Also, the relative ratio of the simian virus 40 large and small T antigens is controlled at the level of lariat branch site utilization (9, 24). Examination of the sequence of the bovine growth hormone intron D reveals nothing apparently unusual, although there is minor departure from the consensus sequence at the splice donor site, which matches in only four of the eight positions. The first adenosine residue is 39 nucleotides upstream of the splice acceptor site. This distance may be near the upper limit of the consensus 20 to 40 nucleotides, although it has been established that the lariat branch site is not always an adenosine residue (8, 14, 15). The homologous adenosine 39 nucleotides upstream of the splice acceptor site has been demonstrated to be the nucleotide used as the lariat branch site in splicing of the human intron D (14). As mentioned previously, introns D of the bovine and human genes possess a considerable degree of similarity (12). Finally, there are two predicted perfect hairpin structures of 9 and 7 bp beginning 49 and 137 nucleotides, respectively, downstream of the splice donor site. The second of these hairpins is predicted by the above-mentioned secondary-structure prediction algorithm (37) to be closely associated with the splice acceptor site, and the 5′ side of the stem bears some similarity (assuming G · U base pairing) to the inverted repeat and other sequences within exon 5 of growth hormone implicated in this study. Whether any of these features, either individually or collectively, contribute to the retention of intron D remains to be determined.

In conclusion, the observation that splicing of intron D from bovine growth hormone mRNA is markedly influenced by sequences within exon 5, and that these influences are specific for intron D of bovine growth hormone, suggests a possible role for this exon-intron interdependence in maintaining a particular steady-state level of intron D-containing mRNA. As discussed previously (13), the translated polypeptide resulting from retention of intron D likely would possess properties differing significantly from those of wild-type growth hormone. At this time there is no known tissue- or develop-specific expression of this alternatively processed growth hormone mRNA. Nonetheless, if it is assumed that the polypeptide resulting from retention of intron D has a physiological function, regulation of alternative processing may still be required to provide appropriate steady-state levels of the protein. This may be an intrinsically regulated mechanism in which the relative ratio of the spliced versus unspliced mRNAs is determined kinetically, reflecting the relative effectiveness of the intron and exon

sequences as a substrate for the splicing machinery. Alternatively, there may be yet undetermined tissues in which growth hormone is expressed in predominantly the form that retains intron D.

ACKNOWLEDGMENTS

We are especially grateful to Edward C. Goodwin for generously sharing his plasmids containing nuclease BAL 31 deletions in exon 5 before publication, for supplying the clone containing deletion of intron D, and for helpful discussions during the course of this study. We also thank Simon Carroll for supplying the prolactin minigene along with corresponding in vitro-synthesized transcripts and to Wessel Dirksen for synthesis of oligonucleotides. We thank Timothy Nilsen, John Nilson, Richard Davis, and Sherron Helms for critical evaluation of the manuscript.

This work was supported by Public Health Service grant DK32770-06 from the National Institutes of Health to F.M.R. R.K.H. was a recipient of a postdoctoral fellowship from the American Heart Association, Northeast Ohio Affiliate Inc. L.L. was a recipient of a Public Health Service medical student short-term summer research fellowship from the National Institutes of Health.

LITERATURE CITED

1. **Aebi, M., H. Hornig, R. A. Padgett, J. Reiser, and C. Weissman.** 1986. Sequence requirements for splicing of higher eukaryotic nuclear pre-mRNA. Cell **47:**555–565.
2. **Berger, S. L., and C. S. Birkenmeier.** 1979. Inhibition of intractable nucleases with ribonucleoside-vanadyl complexes: isolation of messenger ribonucleic acid from resting lymphocytes. Biochemistry **18:**5143–5149.
3. **Breitbart, R. E., and B. Nadal-Ginard.** 1987. Developmentally induced, muscle-specific trans factors control the differential splicing of alternative and constitutive troponin T exons. Cell **49:**793–803.
4. **Camper, S. A., Y. A. S. Yao, and F. M. Rottman.** 1985. Hormonal regulation of the bovine prolactin promoter in rat pituitary tumor cells. J. Biol. Chem. **260:**12246–12251.
5. **Cech, T. R., and B. L. Bass.** 1986. Biological catalysis by RNA. Annu. Rev. Biochem. **55:**599–629.
6. **Cooke, N. E., J. Ray, J. G. Emery, and S. A. Liebhaber.** 1988. Two distinct species of human growth hormone-variant mRNA in the human placenta predict the expression of novel growth hormone proteins. J. Biol. Chem. **263:**9001–9006.
7. **Crenshaw, E. B., III, A. F. Russo, L. W. Swanson, and M. Green.** 1987. Neuron-specific alternative RNA processing in transgenic mice expressing a metallothionein-calcitonin fusion gene. Cell **49:**389–398.
8. **Freyer, G. A., J. Arenas, K. K. Perkins, H. M. Furneaux, L. Pick, B. Young, R. J. Roberts, and J. Hurwitz.** 1987. *In vitro* formation of a lariat structure containing G2′-5′G linkage. J. Biol. Chem. **262:**4267–4273.
9. **Fu, X.-Y., and J. L. Manley.** 1987. Factors influencing alternative splice site utilization in vivo. Mol. Cell. Biol. **7:**738–748.
10. **Furdon, P. J., and R. Kole.** 1988. The length of the downstream exon and the substitution of specific sequences affect pre-mRNA splicing in vitro. Mol. Cell. Biol. **8:**860–866.
11. **Gerke, V., and J. Steitz.** 1986. A protein associated with small nuclear ribonucleoprotein particles recognizes the 3′ splice site of premessenger RNA. Cell **47:**973–984.
12. **Gordon, D. F., D. P. Quick, C. R. Erwin, J. E. Donelson, and R. A. Maurer.** 1983. Nucleotide sequence of the bovine growth hormone chromosomal gene. Mol. Cell. Endocrinol. **33:**81–95.
13. **Hampson, R. K., and F. M. Rottman.** 1987. Alternative processing of bovine growth hormone mRNA: nonsplicing of the final intron predicts a high molecular weight variant of bovine growth hormone. Proc. Natl. Acad. Sci. USA **84:**2673–2677.
14. **Hartmuth, K., and A. Barta.** 1988. Unusual branch point selection in processing of human growth hormone pre-mRNA. Mol. Cell. Biol. **8:**2011–2020.
15. **Hornig, H., M. Aebi, and C. Weissmann.** 1986. Effect of mutations at the lariat branch acceptor site on alpha-globin pre-mRNA splicing *in vitro*. Nature (London) **324:**589–591.
16. **Lathe, R., M. P. Kieny, S. Skory, and J. P. Lecocq.** 1984. Linker tailing: unphosphorylated linker oligonucleotides for joining DNA termini. DNA **3:**173–182.
17. **Law, R., M. D. Kuwabara, M. Briskin, N. Fasel, G. Hermanson, D. S. Sigman, and R. Wall.** 1987. Protein-binding site at the immunoglobulin $\mu_{membrane}$ polyadenylation signal: possible role in transcription termination. Proc. Natl. Acad. Sci. USA **84:**9160–9164.
18. **Leff, S. E., R. M. Evans, and M. G. Rosenfeld.** 1987. Splice commitment dictates neuron-specific alternative RNA processing in calcitonin/CGRP gene expression. Cell **48:**517–524.
19. **Legrain, P., B. Seraphin, and M. Rosbash.** 1988. Early commitment of yeast pre-mRNA to the spliceosome pathway. Mol. Cell. Biol. **8:**3755–3760.
20. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Maniatis, T., and R. Reed.** 1987. The role of small nuclear ribonucleoprotein particles in pre-mRNA splicing. Nature (London) **325:**673–678.
22. **Mardon, H. J., G. Sebastio, and F. Baralle.** 1987. A role for exon sequences in alternative splicing of the human fibronectin gene. Nucleic Acids Res. **15:**7725–7733.
23. **Nilsen, T. W., and P. A. Maroney.** 1984. Translational efficiency of cMyc mRNA in Burkitt lymphoma cells. Mol. Cell. Biol. **4:**2235–2238.
24. **Noble, J. C. S., Z.-Q. Pan, C. Prives, and J. L. Manley.** 1987. Splicing of SV40 early pre-mRNA to large T and small t mRNAs utilizes different patterns of lariat branch sites. Cell **50:**227–236.
25. **Parent, A., S. Zeitlin, and A. Efstratiadis.** 1987. Minimal exon sequence requirements for efficient *in vitro* splicing of mono-intronic nuclear pre-mRNA. J. Biol. Chem. **262:**11284–11291.
26. **Plotch, S. J., and R. M. Krug.** 1986. *In vitro* splicing of influenza viral NS1 mRNA and NS1-β-globin chimeras: possible mechanisms for the control of viral mRNA splicing. Proc. Natl. Acad. Sci. USA **83:**5444–5448.
27. **Reed, R., and T. Maniatis.** 1986. A role for exon sequences and splice-site proximity in splice-site selection. Cell **46:**681–690.
28. **Sharp, P. A.** 1987. Splicing of messenger RNA precursors. Science **235:**766–771.
29. **Solnick, D.** 1985. Alternative splicing caused by RNA secondary structure. Cell **43:**667–676.
30. **Somasekhar, M. B., and J. E. Mertz.** 1985. Exon mutations that effect the choice of splice sites used in processing the SV40 late transcripts. Nucleic Acids Res. **13:**5591–5609.
31. **Tazi, J., C. Alibert, J. Temsamani, I. Reveillaud, G. Cathala, C. Brunei, and P. Jeanteur.** 1986. A protein that specifically recognizes the 3′ splice site of mammalian pre-mRNA introns is associated with a small nuclear ribonucleoprotein. Cell **47:**755–766.
32. **Tuerk, C., P. Gauss, C. Thermes, D. R. Groebe, M. Gayle, N. Guild, G. Stormo, Y. d'Aubenton-Carafa, O. C. Uhlenbeck, I. Tinoco, E. N. Brody, and L. Gold.** 1988. CUUCCG hairpins: extraordinarily stable RNA secondary structures associated with various biochemical process. Proc. Natl. Acad. Sci. USA **85:**1364–1368.
33. **Wieringa, B., E. Hofer, and C. Weissmann.** 1984. A minimal intron length but no specific internal sequence is required for splicing the large rabbit β-globin intron. Cell **37:**915–925.
34. **Woychik, R. P., S. A. Camper, R. H. Lyons, S. Horowitz, E. C. Goodwin, and F. M. Rottman.** 1982. Cloning and nucleotide sequencing of the bovine growth hormone gene. Nucleic Acids Res. **10:**7197–7210.
35. **Woychik, R. P., R. H. Lyons, L. Post, and F. M. Rottman.** 1984. Requirement for the 3′ flanking region of the bovine growth hormone gene for accurate polyadenylylation. Proc. Natl. Acad. Sci. USA **81:**3944–3948.
36. **Zoller, M. J., and M. Smith.** 1984. Oligonucleotide-directed mutagenesis; a simple method using two oligonucleotide primers and a single strand DNA template. DNA **3:**479–488.
37. **Zucker, M., and P. Stiegler.** 1981. Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information. Nucleic Acids Res. **9:**133–148.