Human Growth Hormone Gene and the Highly Homologous Growth Hormone Variant Gene Display Different Splicing Patterns

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Abstract

Stably transfected cell lines containing the normal human growth hormone (hGH-N) and human growth hormone-variant (hGH-V) genes have been established in order to study the expression of these two highly homologous genes. Each gene was inserted into a bovine papillomavirus shuttle vector under the transcriptional control of the mouse metallothionein gene promoter and the resultant recombinants were transfected into mouse C127 cells. The transfected cells containing the hGH-N gene secrete two hGH proteins, 91% migrating at 22 kD and 9% migrating at 20 kD, the same relative proportions synthesized in vivo by the human pituitary. S1 nuclease analysis of mRNA from these cells confirms that 20 kD hGH is encoded by an alternatively spliced product of the primary hGH-N gene transcript in which the normal exon 3 splice-acceptor site is bypassed for a secondary site 15 codons within exon 3. Although the hGH-V gene is identical to the hGH-N gene for at least 15 nucleotides on either side of the normal and alternative exon 3 *AG* splice-acceptor sites, hGH-V synthesizes only a 22-kD protein. Reciprocal exchange of exon 3 and its flanking intron sequences between the hGH-N gene and the hGH-V gene, eliminates the synthesis of the 20-kD protein in both resultant chimeric genes. These results directly demonstrate that both the major 22-kD and the minor 20-kD forms of pituitary hGH are encoded by the alternative splicing products of a single hGH-N gene transcript. This alternative splicing is neither species nor tissue-specific and appears to be regulated by at least two separate regions remote from the *AG* splice-acceptor site.

Introduction

The human growth hormone gene (hGH-N)[1] is 1.65 kilobase pairs (kb) in length and is composed of five exons and four introns (1, 2). This gene is expressed in the pituitary and encodes a 22-kD protein. In addition to this predominant form, ~ 10% of pituitary hGH is present as a 20-kD protein that has an amino acid sequence identical to 22 kD hGH except for a 15 amino acid internal deletion of residues 32 to 46 (3–6). Since a separate gene that might encode this smaller hGH has not been detected (7), the 20-kD hGH was postulated to represent the product of an alternatively spliced hGH mRNA. Exon 2 of the normal hGH gene was postulated to be joined to a splice-acceptor site internal to exon 3, thereby resulting in the 15 amino acid internal deletion. Limited S1 nuclease analysis of pituitary mRNA has supported this alternative splicing hypothesis (1). Although this alternative splicing hypothesis is consistent with the known amino acid structure of the 20-kD protein, fibroblasts transfected with the normal pituitary hGH gene were found to synthesize only the 22-kD protein (8). These results suggest that the 20-kD protein is either the product of an undetected, unique gene, or that the hypothesized alternative splicing of the hGH gene transcript is pituitary specific.

A second hGH gene has recently been identified within the hGH gene cluster on chromosome 17. To distinguish it from the normal hGH-N gene, which encodes pituitary hGH, this second gene has been named hGH-variant (hGH-V). The hGH-V gene differs from the hGH-N gene by 35 scattered base substitutions in the coding region, resulting in 15 amino acid changes including 13 in the mature secreted hormone (2). This gene has been expressed by fibroblasts infected with an SV40-hGH-V recombinant virus as a 22-kD protein at levels comparable to the 22-kD hGH protein (8). The levels of the hGH-V and hGH-N proteins expressed in this system may have been influenced by the adjacent SV40 promoter sequences within the recombinant viral vector. The actual site(s) and level of expression of the hGH-V gene in vivo are now being characterized (9) and the physiologic function(s) of the encoded proteins remain to be defined. The sequences of the hGH-N and hGH-V genes are identical for at least 15 nucleotides on either side of the normal exon 3 *AG* splice-acceptor sites and at the putative alternative acceptor sites within exon 3. Based upon this identity it has been speculated that the hGH-V gene also should encode a 20-kD protein (2). Whether the hGH-V gene does actually undergo alternative splicing has not been previously investigated.

In this study we constructed four stably transfected cell lines which express either the hGH-N gene, the hGH-V gene, or one of two chimeric genes in which exon 3 and its flanking intron sequences have been exchanged reciprocally between the hGH-N and the hGH-V genes. We used these cell lines to define the origin of the 20-kD hGH protein, to determine whether the putative alternative splicing of the hGH-N gene transcript is pituitary specific, to assess whether a parallel pattern of mRNA processing exists for the highly homologous hGH-V gene, and to begin to determine the structural basis for the alternative splicing.

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1. *Abbreviations used in this paper:* BPV, bovine papilloma virus; hGH-N, normal human growth hormone; hGH-V, human growth hormone variant; mMT, mouse metallothionein; nt, nucleotides.

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Methods

Chimeric gene constructions and subcloning into expression vectors. All recombinant manipulations were carried out using standard protocols (10). Restriction and modification enzymes were purchased from either Bethesda Research Laboratories Inc., Gaithersburg, MD or New England Biolabs Inc., Beverly, MA and used as suggested by the supplier. Xho I linkers were purchased from New England Biolabs and were phosphorylated prior to ligation. The isolation of the hGH-N gene and the construction of the hGHd3 gene have been previously reported (11).

To construct hGH-N and hGH-V expression recombinants, the two 2.6-kb Eco RI genomic fragments containing either the hGH-N or hGH-V genes along with ~ 500 bp of 5′ flanking region and 800 bp of 3′ flanking region were separately subcloned into pBR322 and subsequently inserted into a bovine papillomavirus shuttle vector pBPV-MT-Xho I (pBPV-MT-X). This vector contains the entire 7944 bp BPV genome (12, 13) joined to the 2.6 kb "poison-minus" pBR322 sequence (pML2 [14]), and the mouse metallothionein gene (mMT [15]). The naturally occurring Bgl II site in the first exon of the mMT gene (within the 5′ nontranslated region) was replaced with a Xho I linker which then becomes the only Xho I site in the vector (Fig. 1). This vector, constructed by D. Hamer, National Institutes of Health (personal communication), was a generous gift. The hGH-N and hGH-V genes in the pBR322 subclones were digested with Bam HI, which cuts both genes in exon I, one base 3′ to the cap addition site (1, 2), and cleaves the pBR322 vector 375 nucleotides 3′ to the Eco RI insertion site (16). The resulting 2.5-kb fragment (Fig. 1, *top line*) was isolated on an 0.8% agarose gel, recovered from a gel slice by flint silica extraction (17) and then ligated to the Xho I-digested dephosphorylated pBPV-MT-X vector. The recombinants were detected in transformed *Escherichia coli* HB101 by in situ hybridization with a nick-translated ^{32}P-labeled hGH cDNA (18) probe.

The hGH-NV3 and hGH-VN3 genes were constructed as detailed in Fig. 2. The final hGH-NV3 and hGH-VN3 chimeras, containing exon 3 of the hGH-V gene in an hGH-N background or exon 3 of hGH-N in a hGH-V background, respectively, were identified in transformed bacteria by in situ screening with a nick-translated hGH-N exon 3 probe (the Sac I-Sma I fragment, Fig. 2). These chimeric genes were subsequently isolated and inserted into the pBPV-MTX vector as described above for the hGH-N and hGH-V genes. All plasmids were maintained in *E. coli* HB101 under P1 containment conditions.

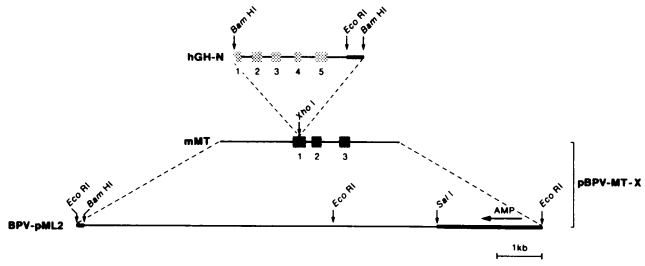

Figure 1. The recombinant expression plasmid pBPV-MT-X-GH-N. The hGH-N gene was inserted into the pPBV-MT-X shuttle vector by fusing the 5′ nontranslated regions of the hGH-N and the mMT genes (both in exon I of the respective genes). The hGH-N gene is in the same transcriptional orientation as the mMT gene so that its expression is under the control of the mMT promoter. Regions of the hGH-N and mMT genes and the BPV and pML2 sequences are labeled. Details of the vector and construction are described in the text. The three mMT exons and the five hGH exons are numbered and shown in light and dark shading, respectively. pML2 sequences are shown as a heavy line and the position and transcriptional orientation of the ampicillin gene is indicated by the horizontal arrow (*AMP*). Relevant restriction sites and a 1-kb scale marker are indicated.

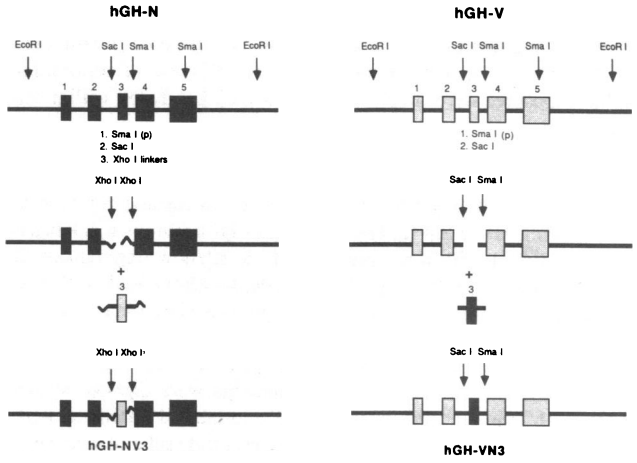

Figure 2. Construction of the hGH-NV3 and hGH-VN3 chimeric genes. Exon 3 was removed from the hGH-N gene and replaced with an Xho I linker to generate hGHd3 as previously described (11) and summarized in the numbered steps at the upper left. Exon 3 of the hGH-V gene was released on a Sac I-Sma I fragment, was ligated to Xho I linkers, and was inserted in the proper transcriptional orientation at the Xho I site of the hGHd3 gene as detailed in the text and shown on the left side. Exon 3 of hGH-N was released on a Sac I-Sma I fragment and was ligated directly into the Sac I-Sma I site of hGH-V as shown on the right side. The exons of the hGH-N gene are shown as black rectangles, those of the hGH-V exons as shaded rectangles, and Xho I linkers as zig-zag lines. The restriction sites relevant to the construction are shown, (*p*) refers to partial digestion.

Cell culture and transfections. The C127 mouse mammary tumor cell line (American Type Culture Collection, Rockville, MD) was maintained at 37°C with 5% CO_2 in Eagles' modified minimal essential medium containing 8% fetal bovine serum (Flow Laboratories, McLean, VA). This cell line was used in all the transfection experiments because expression of the transfected BPV genome gives these cells an easily detectable transformed phenotype (19). Logarithmically growing C127 cells in a 60-mm Petri dish were transfected with 1 to 5 μg of supercoiled plasmid DNA by $Ca_3(PO_4)_2$ coprecipitation followed by shocking the cells with glycerol at 10% final concentration for 1 min (13). Cells were then maintained for 3–5 wk with alternate day media changes until discrete macroscopic foci of swirling, fibroblastlike cells were observed. These transformed foci were isolated using 4 mm cloning rings and transferred to 24-well microtiter plates. Once the cells were confluent, media from each well was assayed by a standard ELISA (20). The primary rabbit anti-hGH sera for this assay was raised by subcutaneous immunization of New Zealand White rabbits with pituitary hGH (kindly provided by S. Raiti, National Hormone and Pituitary Program). The secondary antibody, goat anti-rabbit immunoglobulin, was purchased from Zymed, South San Francisco, CA. The cell lines that secreted immunoreactive hGH were expanded and frozen in multiple aliquots so that fresh cells could be thawed at monthly intervals to minimize drift in the expression of the transfected genes.

Western analysis. Logarithmically dividing cells were incubated overnight in the presence of serum-free media (HL-1 media; Ventrex Laboratories, Inc., Portland, ME). A 25- to 200-μl aliquot of this media was fractionated on a 12% polyacrylamide-SDS gel (21) alongside prestained protein molecular weight size markers (Bethesda Research Laboratories), and was then electro transferred to nitrocellulose paper. Western analysis was carried out according to standard protocol (22) using rabbit anti-hGH sera as the primary antibody, goat anti-rabbit immunoglobulin as the secondary antibody, and iodinated staphylococcus aureus protein (Amersham Corp., Arlington Heights, IL) for

detection of the immune complexes. Band intensity on autoradiographs was measured by soft laser densitometry (Biomed Instruments Inc., Fullerton, CA) with online integrating software. All autoradiographic exposures were timed so that analyzed bands fell within the linear range.

S1 nuclease mapping. Total cellular RNA was isolated from a confluent T75 flask of transfected C127 cells by the method of Strohman et al. (23). The full-length hGH cDNA clone was linearized by digestion with Bam HI or Bgl II. The free 3′ Bam HI terminus was labeled with alpha-[^{32}P]dGTP in the presence of the Klenow fragment of *E. coli* DNA polymerase. The Bgl II site was dephosphorylated at the free 5′ ends with calf intestinal alkaline phosphatase (Bethesda Research Laboratories), followed by labeling with gamma-[^{32}P]ATP in the presence of T4 polynucleotide kinase (Pharmacia Fine Chemicals, Piscataway, NJ). In both cases the labeled fragments were digested subsequently with Hind III; the unique 5′ or 3′ end-labeled fragments were resolved by electrophoresis on a 5% polyacrylamide gel and were electroeluted from the appropriate gel slice. 10 μg of total RNA from the indicated cell lines were hybridized individually to 100 ng of each end-labeled, heat-denatured (100°C for 2 min) DNA probe in 30 μl of hybridization buffer (80% deionized formamide, 400 mM NaCl, 40 mM Pipes, pH 6.4, 1 mM EDTA) for 10 min at 70°C, then at 42°C overnight. The hybridization reaction was directly added to 300 μl of ice-cold S1 nuclease reaction mix (30 mM sodium acetate pH 4.6, 50 mM NaCl, 1 mM $ZnSO_4$, 5% glycerol) containing 50 U of S1 nuclease (Bethesda Research Laboratories) followed by incubation at 37°C for 2 h. The reaction products were precipitated with ethanol and analyzed on a 5% acrylamide 8 M urea gel in parallel with DNA size markers pBR322 digested with Hinf I, end-labeled with ^{32}P. The dried gel was autoradiographed at −70°C on Kodak XAR film in the presence of a Cronex Lightning-Plus intensifying screen.

Results

To facilitate comparative studies of the expression of the hGH-N and hGH-V genes, we established a series of stable cell lines expressing each of these genes under the control of the mMT promoter. This was done by transfecting C127 cells with the recombinant plasmids pBPV-MT-X-GH-N (Fig. 1) and pBPV-MT-X-GH-V. Two transformed cell lines, one expressing hGH-N (CM-GH) and one expressing hGH-V (CM-V), were selected for study. A control cell line transfected with the pBPV-MT-X vector alone (CM) was isolated in parallel. The immunoreactive proteins secreted into the media by the CM-GH and CM-V cell lines were studied by Western analysis using a rabbit polyclonal hGH antiserum. The results of this analysis are shown in Fig. 3. The control cell line (lane *1*), CM, secretes no immunoreactive GH protein. Both the hGH-N and hGH-V genes encode the expected 22-kD proteins (lanes *2* and *3*, respectively) which comigrate with authentic pituitary hGH, while a smaller protein of 20 kD is present in the CM-GH but not the CM-V lane. By densitometric analysis of the autoradiographs the 20-kD protein represented 9% of the total hGH secreted into the media by the CM-GH cells (lane *2*). The bands above the 22-kD hGH-N and hGH-V bands may represent posttranslational modifications and are not further characterized in this report. The hGH-N therefore secretes a 20-kD protein that is not secreted by the highly homologous hGH-V gene.

To confirm that the 20-kD hGH protein represents the product of an alternatively spliced hGH mRNA, the mRNA from the CM-GH cell line was isolated and mapped by S1 nuclease analysis. A previous S1 analysis of hGH mRNA (1) was expanded by using two different S1 probes, one 5′-end labeled and the other 3′-end labeled, in order to define both the

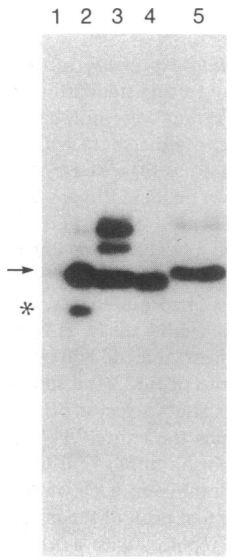

Figure 3. Expression of the transfected hGH-N, hGH-V, hGH-NV3, and hGH-VN3 genes in C127 cells. Cell lines CM, CM-GH, CM-V, CM-NV3, and CM-VN3 (lanes 1–5) were grown overnight in serum-free media. The media was then applied to a SDS-polyacrylamide gel and analyzed by Western blotting using hGH antiserum. The amounts of media loaded on the gel lanes 1–5 (50, 20, 100, 40, and 200 μl, respectively) were adjusted to give comparable signals in the positive lanes. The size of the proteins detected on the autoradiograph are determined by comparison to known molecular size standards run on a parallel lane (not shown), the size and position of which are indicated. The position of 22 kD pituitary hGH, as determined by Coomassie Blue staining, is indicated by the arrow. The position of the 20-kD hGH band is indicated by the asterisk.

5′ and 3′ ends of the internal deletion in this shorter mRNA. The probes used and the strategy followed are shown in Fig. 4, panel *C*. To generate a marker band delimiting the 3′ boundary of hGH exon 2, mRNA from cells transformed with a chimeric gene in which the third exon of hGH has been replaced by the nonhomologous third exon of the rat prolactin gene, hGHP3 (11), was mapped with the 5′ probe (Fig. 4, *A*, lane *4*). The results of the S1 nuclease analysis on RNA from CM-GH and CM (Fig. 4, *A* and *B*, lanes *3* and *2*, respectively) demonstrate that two protected hGH species are present in the CM-GH mRNA; the major hGH mRNA species is perfectly homologous to the full-length hGH cDNA probe, while the second, less abundant species, diverges from the hGH mRNA sequence in a discrete region which correlates in its 5′ and 3′ borders with the proposed alternative splicing site of the hGH-N gene. By densitometric analysis of the autoradiographs, the divergent species constitutes 10% of the total hGH mRNA detected in this experiment, consistent with the abundance of the 20-kDa protein in the media (Fig. 3, lane *2*). The 22 and 20 kDa proteins are therefore translated from two unique mRNAs processed from the single hGH-N gene transcript.

In order to characterize the structural basis for the difference in the splicing patterns between the hGH-N and the hGH-V transcripts, exon 3 of hGH-N was removed (hGHd3, 11) and replaced by exon 3 of the hGH-V gene (hGH-NV3); reciprocally exon 3 of hGH-V was removed and replaced by exon 3 of the hGH-N gene (hGH-VN3). The construction of both hGH-NV3 and hGH-VN3 are detailed in Fig. 2. Exon 3 along with 118 flanking bases of intron B and 57 flanking bases of intron C was removed from both the hGH-N and hGH-V genes on a Sac I-Sma I restriction fragment. Xho I linkers were ligated to hGH-V exon 3 which was reinserted into the Xho I site of hGHd3. In the hGH-VN3 construct, no linkers were used as the hGH-N exon 3 was inserted directly into the Sac I-Sma I sites of hGH-V. The structure of the resultant chimeric

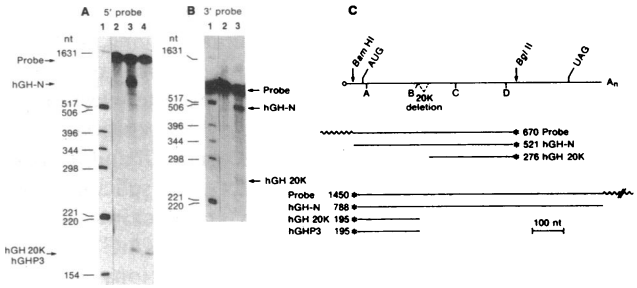

Figure 4. S1 nuclease mapping of mRNA from transfected cell lines. RNAs isolated from CM, (*A* and *B*, lanes *2*), CM-GH (*A* and *B*, lanes *3*), and CM-P3 (*A*, lane *4*) cells were analyzed by hybridization to 670 and 1,450 bp probes labeled with ^{32}P at the ends indicated by asterisks (*C*). This was followed by S1 nuclease digestion. Lanes *1* in A and B, contain end-labeled molecular weight markers, pBR322 digested with Hinf I. The positions of the end-labeled probes, the hGH-N and hGH 20-kD fragments specifically protected from S1 nuclease digestion are indicated by arrows to the left of A (5'-labeled probe) and to the right of B (3'-labeled probe) and their positions in hGH mRNA are diagrammed in C. The diagram of the hGH-N mRNA at the top of C shows the position of the two restriction sites used in the end-labeling (Bam HI and Bgl II), the position of the 5' cap (○), the initiation codon (AUG), the termination codon (UAG), the poly A tail (A_n), the site at which each of the four introns (A–D) interrupt the coding region in the hGH-N gene and the position of the predicted internal deletion in the alternatively spliced hGH-N mRNA (dashed line labeled "20K deletion"). The wavy lines in C indicate pBR322 vector sequences included in the original probe fragment. The diagram in *C* is drawn to scale, as indicated by the 100 nucleotide (*nt*) marker.

genes maximally resembles the two natural genes. The resultant pBPV-MT-X-NV3 and pBPV-MT-X-VN3 chimeric plasmids were transfected into mouse C127 cells. Transformed foci were identified and isolated. Two cell lines were selected, CM-NV3 and CM-VN3. Proteins secreted from these cell lines were analyzed by Western blotting. Both contained a predominant 22-kD band and lacked the 20-kD product (Fig. 3, lanes *4* and *5*). The absence of the 20-kD protein was confirmed by prolonged overexposure of this autoradiograph (data not shown).

Discussion

The experiments described in this report were designed to address a number of questions regarding hGH expression. The first question was, does the hGH-N gene encode 20-kD pituitary hGH? Evidence cited in the Introduction suggests that this is the case, but this conclusion was questioned when others were unable to detect the 20-kD hGH protein in cells expressing a transfected hGH gene (8). In the experiment shown in Fig. 3, we demonstrate that a 20-kD hGH protein is, in fact, encoded by the single transfected hGH-N gene. In addition, the proportion of 20-kD hGH encoded by the transfected hGH gene is the same as that detected in the pituitary by Lewis et al. (5). We substantiate that this protein represents the previously described 20-kD internal deletion protein by demonstrating that the CM-GH cells contain both normal hGH mRNA and an mRNA with a 45-base deletion at the 5' end of exon 3 encoded in the same proportion as the 22- and 20-kD proteins are expressed. The expression of the 20-kD hGH alternative splicing product by an hGH-N gene transfected into a mouse mammary tumor cell line further demonstrates that this splicing pattern is neither pituitary nor species specific. It is not clear why this 20-kD hGH was not seen previously when the hGH-N gene was introduced into a monkey kidney cell line by infection with a recombinant SV40 viral vector (8). We have repeated those experiments using the same pSV-hGH recombinant virus infection of the monkey kidney cells and have found expression of both 22- and 20-kD hGHs at the same relative levels that we report here using stable transformation of BPV recombinants and C127 mouse cells (unpublished data). The difference between these results and the previous negative result suggests that as yet unidentified factors in the experimental approach may modulate the variable splicing of the hGH-N transcript or its detection. Based on our data, we conclude that the alternative splicing pattern and relative concentrations of 20- and 22-kD hGH are constitutive features of hGH-N gene expression.

In order to characterize the structural basis for the alternative splicing of the hGH-N gene transcript, we compared it to the splicing pattern of the homologous hGH-V gene transcript. The hGH-V gene only expresses a 22-kD protein in contrast to the 22- and 20-kD proteins expressed by hGH-N. This absence of alternative splicing between exons 2 and 3 of the hGH-V transcript is unexpected on the basis of the similarity of hGH-N and hGH-V DNA sequences. The hGH-V and hGH-N genes share identical sequences for more than 15 bases on either side of the normal and alternate *AG* splice-acceptor sites in exon 3 (Fig. 5, sites B and B', respectively). This region includes the 10 nucleotides of the acceptor site consensus sequence which surrounds the invariant *AG* (24). The alternative splicing pattern is lost when this region of the hGH-N gene is replaced by the corresponding region of the hGH-V gene (hGH-NV3). This result suggests that sequences in the 3' end of intron B, or in exon III outside the region of the *AG* and its immediate consensus region contribute to the usage of the alternative splice-site. To determine whether the entire controlling signal is located in this cassetted region or whether an additional more remote region is involved, we tested the splic-

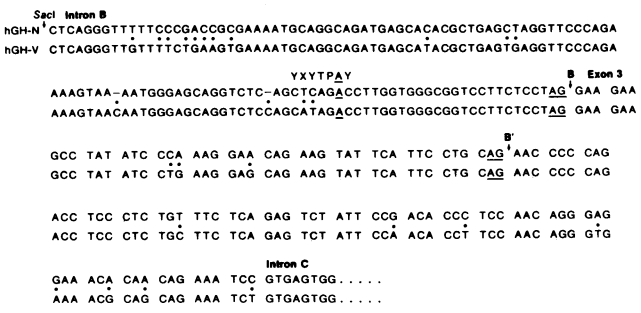

Figure 5. Comparison of the hGH-N and hGH-V gene sequences in the region surrounding the variable splicing site. The sequences of the hGH-N and the hGH-V genes (2) extending from the Sac I site in intron B (see Fig. 2), through exon 3 and into intron C are compared. The positions of base mismatches are identified with a dot, the AG of the normal and alternative splice-acceptor sites are underlined and labeled B and B', respectively. The A of the predicted lariat branch point is underlined. Its position was based on the branch point consensus sequence, YXYTPAY, where *Y* is a pyrimidine, *X* is any base, and *P* is a purine (25).

ing pattern of the reciprocal construction in which exon 3 of the hGH-N gene replaces the corresponding region of the hGH-V gene (hGH-VN3). If the entire controlling signal were in this region, hGH-VN3 should encode both the 22- and 20-kD proteins. This was not the case as no 20-kD band was seen, even after overexposure of the autoradiographs. Therefore the cassetted region that contains both of the potential splice sites B and B′ does not by itself direct the usage of the B′ site because placing the entire exon 3 and flanking introns of hGH-N in an hGH-V background does not reconstitute the use of the alternative B′ site. These results suggest that the splice-site selection at the intron 2/exon 3 junction of the hGH-N transcript is directed by at least two regions remote from the acceptor site itself. One region, the proximal signal, must be located within the cassetted exon 3 fragment because hGH-NV3 does not alternatively splice; the other region, the distal signal, must be located outside of the cassetted exon 3 fragment because hGH-VN3 is unable to alternatively splice.

A number of structural differences between the two GH genes might account for the difference in their splicing patterns. A functionally important structural component of the splicing reaction is the formation of a phosphodiester bond between the 5′ terminus of the intron and an adenosine, the lariat branch point, which is embedded in a consensus sequence and is located between 20 and 40 nucleotides 5′ to the *AG* splice acceptor site (25). The most likely position of the normal splicing site branch point in the hGH gene (both N and V) is noted on Fig. 5 by the YXYTP*A*Y where *A* is the putative lariat branch point. The two GH genes differ by two bases in a weakly conserved region −3 and −4 bases from the probable *A* branch point. While such a minor difference has not previously been demonstrated to dictate the utilization of a splice site, it is possible that these base differences could result in a marginal difference in the relative strengths of the normal splice-acceptor sites of two otherwise homologous genes. In addition there are several differences between the primary structures of the two genes 5′ of the *AG* which serves as the alternative splice-acceptor site for hGH-N (Fig. 5, site B′). A difference of particular interest is the A to G change at position −20 relative to the B′ site. This *A* is in the proper position and is surrounded by the appropriate consensus sequence to serve as a lariat branch point for the B′ splice. While a number of studies predict that alterations in the lariat region do not play a major role in splice-site selection (for review see 26), this possibility must be considered in the comparison of two otherwise highly similar genes with different splicing patterns. These branch-point differences would be postulated to represent the proximal signals controlling the alternative splicing pathway.

In addition to these proximal interactions, sequences outside the splice-site consensus regions may also alter the availability of the normal exon 3 acceptor site in hGH-N by sequence specific mRNA-protein or mRNA-RNA interactions (27), or by formation of secondary structures in the hGH-N primary RNA transcript that are not present in the hGH-V primary transcript. Stem and loop regions of secondary RNA structures have been demonstrated to sequester entire exons or specific splice junctions and decrease their utilization in synthetic mRNAs in in vitro splicing systems, as well as during transient expression of transfected genes in intact cells (28). However, in these experiments perfectly matched regions of base-pairing and secondary structure were created by introducing two 105-bp inverted repeats. It remains to be seen whether shorter, less perfectly base-paired regions as seen in natural genes can have a similar effect upon splicing. Such an interaction might account for the apparent contribution of two separate proximal and distal regions to splice-site selection in the hGH-N gene. To pursue this possibility, the position of the distal controlling region must be defined in detail and it must be determined whether it exerts its effect upon splicing independently or via an interaction with the more proximal signal. Analysis of the splicing products of other hGH-N/hGH-V chimeric genes together with site-specific alterations in their structures may clarify this issue.

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