Molecular Forms of Human Rhodopsin Kinase (GRK1)*

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The G protein-coupled receptor kinases (GRKs) are critical enzymes in the desensitization of activated G protein-coupled receptors. Six members of the GRK family have been identified to date. Among these enzymes, GRK1 (rhodopsin kinase) is involved in phototransduction and is the most specialized of the family. GRK1 phosphorylates photoactivated rhodopsin, initiating steps in its deactivation. In this study, we found that human retina expressed all GRKs except GRK4. Based on results of molecular cloning and immunolocalization, it appears that both rod and cone photoreceptors express GRK1. This conclusion was supported by the cloning of only GRK1 from cone-dominated chicken retina. Human photoreceptors also transcribe a splice variant of GRK1, which differs in its C-terminal region next to the catalytic domain. This novel variant, GRK1b, is produced by retention of the last intron. mRNA encoding GRK1b is exported to the cytosol; however, the level of the protein is relatively low compared with GRK1 (now called GRK1a), and GRK1b appears to have very low catalytic activity. Thus, these studies suggest that rods and cones, express the same form of GRK1.

Desensitization of G protein-coupled receptors is mediated, at least in part, by a family of Ser/Thr kinases called GRKs1 (1). Distinct properties set these enzymes apart from other protein kinases, including (a) broad and overlapping substrate specificities that are, however, restricted to ligand-activated G protein-coupled receptors and (b) complex interactions with the receptor that involve low affinity binding of GRKs to the region of the receptor that is phosphorylated and high affinity, multipoint interactions of GRKs with cytoplasmic loops of the receptor. To date, six members of the GRK family have been cloned from vertebrate species and sequenced. The overall catalytic properties of these enzymes are 53–93%, with the lowest sequence homology between group I and group II (1, 2). In addition, four splice variants (α, β, γ, and δ) of GRK4 with different N- or C-terminal regions were found primarily in the testis (3, 4), and GRK6 may exist in two splice forms (5). In vitro, of the four variants of GRK4, only the longest form, GRK4, phosphorylates the model substrate, Rho* (3). This suggests that alternative splicing may be one of the mechanisms for generating GRK isoforms with different specificities. This alternative splicing among the members of the GRK family might be an important diversification mechanism, because only six members have been found so far, whereas hundreds of G protein-coupled receptors are subject to receptor phosphorylation.

Diverse mRNA species are produced by alternative splicing. Splice variants can be generated by several mechanisms, including exon skipping, alternative selection of exons, differential usage of splicing sites, and intron retention. Many splice variants have different tissue or cellular localizations, perform different physiological functions, and are differently regulated. Some of the variants have different sequences in the protein coding region, whereas others differ in their 5′- or 3′-untranslated regions. These untranslated regions frequently contain regulatory elements for transcription, translation, and mRNA stability (6). In rod photoreceptors, Rho* triggers a phototransduction cascade through the activation of a G protein (Gt, also called transducin), leading to an increase in cGMP phosphodiesterase activity. The hydrolysis of intracellular cGMP by phosphodiesterase leads to the closure of cGMP-gated channels in the plasma membrane and hyperpolarization of the photoreceptor cells. The quenching of Rho* is initiated by its phosphorylation, catalyzed by GRK1, and is followed by the binding of the regulatory protein, arrestin, to the phosphorylated Rho* (2). The role of GRK1 in the regulation of phototransduction was further defined by its role in Oguchi’s disease, a special form of congenital night blindness (7–9). The effects on human vision of a mutation in the GRK1 gene causing Oguchi’s disease, was recently investigated in detail. A slowing of rod and cone deactivation kinetics in the homozygote was detected by electoretinography. However, phosphorylation of Rho* appears not to be involved in the regulation of the initial catalytic properties of Rho*. Cones may rely mainly on regeneration for the inactivation of photolyzed visual pigment, but GRK1 (or its cone homolog) also contributes to cone recovery (9).

Phototransduction in rods and cones differs in electrophysiological response kinetics and sensitivity partly because of the differences in cell-specific subsets of phototransduction proteins. Due to the paucity of cones and the difficulties in their isolation from mammalian retina, cone phototransduction is less well understood at the biochemical level. Molecular cloning of cone phototransduction proteins has been successful, includ-
ing cloning of the red/green/blue visual pigments (10), cone G, α, β, and γ subunits (11–13), cone photophaeisterase α and γ subunits (14, 15), cone arrestin (16), and the α subunit of the cGMP-gated cation channel (17). Several phototransduction proteins are present in both rods and cones, including retinal guanylate cyclase 1 (18), guanylate cyclase-activating proteins (GCAP1 and GCAP2) (19, 20), and recoverin (21). GRK1 has also been localized in both bovine rods and cones using polyclonal antibodies, suggesting that cones may contain either GRK1, its splice form, or a closely related homolog (22).

In this study, using a combination of biochemical and immunochemical methods, we found that GRK1 is expressed in rods and cones and that human and chicken retinas contain GRK1 and an alternative spliced form, GRK1b, which retains the last intron. GRK1b is not a ccone-specific splice variant and appears to have low catalytic activity.

EXPERIMENTAL PROCEDURES

Materials—A chicken retinal cDNA library was provided by Dr. S. Semple-Rowland (University of Florida, Gainesville, FL). Human eyes were obtained from the Lions’ Eye Bank (University of Washington, Seattle, WA), and chicken eyes were obtained from Mr. Wendell Luse of ACME Poultry Co., Inc. (Seattle, WA).

Cloning of GRKs from the Fovea Region of the Human Retina—Human fovea tissue punches were taken from 22 human retinas using an 18-gauge needle. Messenger RNA was isolated (FastTrack2, Invitrogen), and reverse transcription-PCR was performed as described (23). The degenerate oligonucleotide primers were designed according to the conserved sequences in the catalytic regions of GRKs (2). The primer pairs used in the first round of PCR were as follows: XZ-1 (forward, 5'-TACGAATTCAC(A/C/T/G)GG(A/C/T/G)AA(A/G)CT(A/C/T-G/TA/T/GC/CT/G-3') and XZ-2 (reverse, 5'-ATCAAGCTTCTTCATCAACG/G-A/G/CC/GCTAT/GA/G/T/GC/GTC/G-5') and XZ-3 (forward, 5'-GGG/GA/C/T/G/TA/G/CT/T/GC/CT/GAG/G/GA/G/GA/G/T/G-5') and XZ-4 (reverse, 5'-AGAAGCTC/GA/CAG/GTGA/C/TG/GA/GAT/GG-3') and XZ-1 and XZ-4; or XZ-3 and XZ-2. The first-round of PCR contained 10 nmol Tris/HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl2, 0.2 mM dNTPs, ~10 ng of cDNA, and 1 μM primers. The samples were heated to 94 °C for 5 min, followed by the addition of 5 units of Taq DNA polymerase (Promega). The reactions were then cycled 30 times (94 °C for 45 s, 60 °C for 1 min, and 72 °C for 1 min) and amplified in a PCR machine. The PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. The band corresponding to the GRK1b band was excised, dissolved in 6 M guanidinium thiocyanate, and counted in a scintillation counter. The activities of GRK1a versus GRK1b were calculated as the ratio of the radioactivity associated with the GRK1b band to the radioactivity associated with the GRK1a band, taking the molecular weight differences of the PCR products into consideration. Primer b (as in genomic cloning) and primer e were used for GRK1a, primer b and primer d were used for GRK1b, and primers FH-13 (5'-CTAAGCTTCAATCTTGTGAGGACTGGATC-3') and FH-17 (5'-AGCCTGGTCCTCAAGGGGAA-3') were used for GCAP1.

In Vitro Translation of GRK1a and GRK1b—Full-length sequences of GRK1a (1,692 bp) and GRK1b (3,6 kb, containing intron 6) were cloned into pGEM-T Easy (Promega). The plasmid DNA was purified through several steps under RNase-free conditions as described below. DNA was isolated using a Qiagen spin miniprep kit (Qiagen), passed through several steps under RNase-free conditions, and precipitated by ethanol, then suspended in diethyl pyrocarbonyl-treated water. The in vitro transcription/translation reaction was carried out using a Tnt T7-coupled reticulocyte lysate system (Promega) according to the manufacturer’s protocol. Briefly, equal molar concentrations of circular template DNA of GRK1a (1 μg) and GRK1b (1.8 μg) were added to the reaction mixture (total 50 μl) containing 25 μl of rabbit reticulocyte lysate, 1 μl of provided amino acid mixture, 1 μl of RNAse inhibitor, and 1 μl of T7 RNA polymerase (Promega). After 2 h at 30 °C, the samples were mixed with 1% SDS and 2 μl of β-mercaptoethanol, heated to 100 °C for 5 min, and centrifuged at 86,000 × g for 30 min. The proteins were separated on a 10%, 1.5-mm thick SDS-polyacrylamide gel and transferred to a polyvinylidene membrane (Millipore) at 90 V for 1.5 h. The translational products were detected by immunoblotting using D11 anti-GRK1 monoclonal antibodies (1.5 mg/ml at diluted 1:10,000). GRK1a activity was measured as described previously (22).

In Situ Hybridization—Human retinas were fixed for 6 h and stored at −20 °C in methanol until use (20). The transcription template was a cDNA fragment encompassing bases 1,500–1,890 of the human GRK1b sequence cloned into pBluescript. The digoxigenin-labeled probes were generated from linearized plasmid DNA using T3 RNA polymerase for the antisense probe and T7 RNA polymerase for the sense probe (Ambion). Both probes were hydrolyzed with 60 μg Na2CO3, 40 μM NaHCO3, and 80 μM dithiothreitol at 60 °C for 45 min to reduce the probe length to 150–250 bases. In situ hybridization was performed as described previously (20).

Expression and Purification of Human GRK1 in Bacteria—Partial or full-length sequences of GRK1a and GRK1b cDNAs were cloned into pQE30 (Qiagen). The plasmid DNA was transformed into Escherichia coli strain M15 (Qiagen) for protein expression. Protein expression and purification were carried out according to the protocol provided by the manufacturer (Qiagen). The purity in SDS-polyacrylamide gel electrophoresis of His-tagged recombinant proteins was greater than 80%.

Anti-human GRK1a and GRK1b Antibodies—The bacterially expressed, full-length human GRK1a was dialyzed against 70 mM sodium phosphate buffer (pH 7.5) and injected into mice with Ribi adjuvant (Ribi ImmunoChem Research, Inc.). Two monoclonal antibodies were produced according to standard procedures (27): G8 (C-terminal speci-
ficity; see Fig. 1) and D11 (N-terminal specificity; see Fig. 1). Monoclonal antibodies were purified using protein A-Sepharose (Pharmacia Biotech Inc.). A bacterially expressed C-terminal fragment of GRK1b (residues 463–598) was used to immunize rabbits to generate a polyclonal antibody (Cocalico Biologicals, Inc.). The anti-human GRK1b polyclonal antibody (UW54) was purified using antigen coupled to CNBr-Sepharose.

**Immunocytochemistry**—The human sections were processed as in the single labeling experiments as described previously (20). For double labeling, tissue sections were first incubated with primary antibodies to GRK1 (G8) and red/green cone opsin (JH492) or blue cone opsin (JH455) (28), followed by secondary Cy-3-conjugated goat anti-rabbit IgG and Cy-2-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.).

**RESULTS**

**GRKs in Human Retinal Fovea**—Several approaches were employed to explore the presence of different forms of GRKs in human retina, especially the existence of cone-specific kinases. For example, a combination of oligonucleotide primers and PCR using freshly prepared cDNA as well as screening of human and bovine retinal cDNA libraries with the bovine GRK1 probe yielded only GRK1 (Rho kinase). Because the human retina is rod-dominant, with 95% rod and 5% cone photoreceptors (29), these methods could have inherent problems in detecting rare cone kinase in the presence of relatively large amounts of rod GRK1. To enrich with cDNA encoding putative cone kinase, 18-gauge punches were taken from human retinas around the fovea that contained a higher ratio of cone to rod cells in addition to the cells of the neuronal retina. mRNA was isolated and reverse-transcribed and followed by amplification of the cDNA from highly conserved catalytic regions using degenerate oligonucleotide primers designed to hybridize with all GRK. Among the 41 clones sequenced, 22 matched the published sequence of GRK1 (2, 24, 30), 15 matched GRK2/3, which have identical sequence in the chosen fragment of the catalytic region (31, 32), 2 matched the sequence of GRK5 (33, 34), and 2 encoded GRK6 (35). No GRK4 sequence (4, 36) was identified in this cDNA. Despite the fact that different GRKs could be amplified from this cDNA, we were unable to detect any homolog of GRK1, suggesting that the human retina contains one visual pigment kinase. Alternatively, rod and putative cone kinases are identical in the catalytic regions defined by the degenerate oligonucleotide primers.

**Localization of GRK1 in Human Retina**—To localize GRK1 in the human retina, monoclonal antibodies were raised against bacterially expressed kinase. Two antibodies were selected for their recognition of the N- (D11) and C-terminal (G8) sites (Fig. 1). Retinal flat mount immunolocalization with G8 monoclonal antibody showed intense staining of cone and rod outer segments throughout the retina (Fig. 2). The immuno- staining was blocked by preincubation of the antibody with recombinant kinase. Immunofluorescence microscopy of human retina with the monoclonal antibody against the C-terminal domain of the kinase revealed that GRK1 was present mainly in the cone outer segments and, to a lesser degree, in rod outer segments. Weak labeling was found in somata and synaptic terminals of the cones and the inner segments of rods (Fig. 3). The immunolabeling was abolished by preincubation of the antibody with bacterially expressed GRK1 (Fig. 3D). In double-labeled sections of human retina, GRK1 was localized to cone outer segments (Fig. 3, A and D), including those whose outer segments were reactive with anti-red/green (Fig. 3, B and C) and blue cone opsins (Fig. 3, E and F). Identical localization of GRK1 was obtained using D11 monoclonal antibody with a specificity toward the N-terminal GRK1 (data not shown), polyclonal antibodies raised against recombinant GRK1 (data not shown), and native GRK1 (22). Thus, the immunostaining was indistinguishable using antibodies of different specificity. These results support the idea that the same kinase may be present in rod and cone cells. It appears that GRK1 is highly abundant in all classes of cone cells.

**A Splice Variant of Human GRK1-containing Intrinsic Sequence**—To identify novel forms of GRK1 from human retinal cDNA, RACE PCR and primers derived from the catalytic region were used to amplify the 3′ and 5′ regions of the kinase. The RACE products were cloned into pCR2.1 and sequenced. 5′-RACE PCR yielded identical clones to human GRK1 (23). From 24 clones derived from the 3′-RACE PCR, 16 clones hybridized with the catalytic region but not with the C-terminal region of GRK1 probes on Southern blots. Since there is only one GRK1 gene in the genome (24), this latter product, named GRK1b (the original GRK1 is now named GRK1a) might be a splice variant of GRK1. This form was observed not only by reverse transcription-PCR, but it was found also by screening the retinal cDNA library (data not shown and Ref. 24). To investigate the molecular structure of the GRK1b tran-
script, human GRK1 genomic DNA was analyzed using a genomic clone containing exon 4 to 7 (G2) (24). The sizes of introns 4, 5, and 6 were identified using a PCR technique (4) (Fig. 4A). Employing PCR primers residing at different exons and introns, it was determined that the GRK1b transcript was identical to GRK1a, except that it retained the last intron, intron 6 (Fig. 4B). In addition, the sequence of intron 6 was identical with the 3' end of GRK1b. Within the intron 6 sequence, there was a stop codon found ~300 bp from the catalytic region (Fig. 5). GRK1b was not an amplification artifact of genomic DNA because the PCR primer pair b and e amplified an 11-kb fragment from the genomic DNA but only 650 bp (corresponding to GRK1a) and 2.4 kb (corresponding to GRK1b) fragments from cDNA (Fig. 4B). Using PCR and pairs of primers a and e and a and d, we have amplified the full-length coding sequence of both GRK1a and GRK1b (Fig. 4, lower panel). All the PCR products from cDNA were sequenced, and their identity to the PCR products from genomic DNA was established by Southern blotting. These results demonstrate that human GRK1a has a splice variant, GRK1b, which retains the last intron in its mRNA.

Radiometric quantitative PCR was performed on cDNA derived from four human retinas to investigate the abundance and prevalence of GRK1b. GRK1a and GRK1b (650- and 740-bp products, respectively) were amplified in the same PCR using primers b and e and b and d (Fig. 6, inset). Next, GRK1a and
GRK1b were amplified separately with an amplification of a fragment of GCAP1 (19) as an internal control (generated a 200-bp product) (Fig. 6, lower panel; see also “Experimental Procedures”). Representative results from four individuals are shown in Fig. 6. The relative abundance of GRK1b over GRK1a was calculated as the ratio of GRK1b cpm to GRK1a cpm, taking the molecular weight difference of the two PCR products into consideration. By radioactivity measurements, the GRK1b level is between 20 and 80% of the GRK1a level in all cases. This result shows that GRK1b transcript is prevalent, variable, and abundant in humans.

Localization of mRNA Encoding GRK1b—In situ hybridization using human tissue and digoxigenin-labeled antisense and sense probes encoding the sequence of the 5'-terminal part of intron 6 was employed to determine if the mRNA of GRK1b has nuclear or ribosomal localization. In human retina, cells in the outer nuclear layer were specifically labeled with the antisense probe (Fig. 7A), whereas no hybridization signal was produced by the sense probe (Fig. 7B). The most intense staining was found in the cone and rod inner segments. Due to the size of the probes (300 bp), however, some nuclear DNA was also nonspecifically stained. This indicates that the amplification of GRK1b transcript does not result from genomic DNA contamination in the cDNA preparation.

GRK1b were amplified separately with an amplification of a fragment of GCAP1 (19) as an internal control (generated a 200-bp product) (Fig. 6, lower panel; see also “Experimental Procedures”). Representative results from four individuals are shown in Fig. 6. The relative abundance of GRK1b over GRK1a was calculated as the ratio of GRK1b cpm to GRK1a cpm, taking the molecular weight difference of the two PCR products into consideration. By radioactivity measurements, the GRK1b level is between 20 and 80% of the GRK1a level in all cases. This result shows that GRK1b transcript is prevalent, variable, and abundant in humans.

To detect GRK1b in human retina, a specific antibody was raised against the unique C-terminal region. The antibody recognizes bacterially expressed, full-length and C-terminal fragments of GRK1b and detects small amounts of the splice form in the human retina (Fig. 1C). Unfortunately, the antibody is low titer and produced high background, making it unsuitable for immunolocalization of the splice form.

The monoclonal antibody D11, which is specific against the N-terminal region that is common to GRK1a and GRK1b, failed to detect significant amounts of the splice form in partially purified preparations of GRK1 (Fig. 1B) or in retinal extracts (data not shown). This low level of GRK1b did not result from possible abnormal structure of the transcript, since GRK1b can be translated in an in vitro translation system, as shown in Fig. 1D. These results suggest, however, that the GRK1b transcript is formed as a result of alternative splicing and that the GRK1b mRNA may be involved in translational regulation or that GRK1b protein is unstable in the human retina. Due to postmortem effects, human retina is not suitable for rod outer segment preparations. Thus, the localization of GRK1b in the cell body or outer segments of photoreceptors is, at the present time, uncertain. Furthermore, the recombinant GRK1b, when expressed in the in vitro expression system, had <5% light-dependent Rho phosphorylating activity as compared with GRK1a assayed in identical conditions.
The cone-dominant chicken retina has been successfully used to clone the cone-specific α-subunit of the cGMP-gated channel (17). Using this strategy, we cloned only one form of GRK1 from chicken retina (accession number AF019766). Immunocytochemistry using G8 monoclonal antibody and chicken retinal section showed immunoreactivity in both rod and cone cells (data not shown). GRK1 has been shown to be present in mammalian pineal glands, which express both Rho and blue cone pigment (23). A pineal-specific opsin, pinopsin, as well as cone opsins, but not Rho, have also been found in chicken pineal (37, 38). We investigated the presence of GRK1 in the chicken pineal by reverse transcription-PCR. Therefore, despite lacking Rho, chicken pineal gland expresses retinal GRK1. A detailed account of cloning and localization will be given elsewhere.3

DISCUSSION

GRKs in the Human Retina—Among the GRK genes, GRK1, GRK2/3, GRK5, and GRK6 were found to be transcribed in human retina. In double immunolocalization experiments, GRK1 was found to be present in all photoreceptors, including red/green and blue opsin-containing cones. Similar results were found using two anti-GRK1 monoclonal antibodies specific for N- or C-terminal regions (Figs. 1–3). In chicken retina, GRK1 immunolocalizes to all photoreceptors, suggesting that the same kinase is present in these cells. The localization of other GRKs in the retina was not investigated. Although we cannot completely exclude the existence of a novel cone-specific kinase, our data suggest, however, that both rods and cones of human and chicken retinas express the same photoreceptor-specific kinase, GRK1. This conclusion is based on the following evidence. (a) Screening human and chicken retinal libraries yielded one form of the enzyme, (b) PCR with degenerate oligonucleotides derived from the catalytic region of GRKs yielded only one form of GRK1 even though distantly related GRK2/3 was detected, (c) freshly prepared mRNA from cone-rich retina (chicken) and cone-enriched fovea of human retina yielded one GRK1 with reverse transcription-PCR and RACE methods, (d) one photoreceptor kinase is present in chicken and mammalian pineal gland (23), (e) the kinase was immunolocalized to both rod and cone cells using specific antibodies (this study and Ref. 22), and (f) lack of a novel sequence derived from human retina deposited in the EST data base. However, it is possible that lower vertebrates have more than one kinase, as they have more than one recoverin, for example (39).

Splice Form of GRK1—Gene expression is controlled in part by mRNA processing. Alternative splicing of nuclear mRNA (pre-mRNA) occurs in at least one out of 20 genes (6). Sequences that are essential for intron removal are limited to the intron/exon borders (40). In some cases, intron retention is believed to result from suppression of the utilization of both 5’ and 3’ splice sites on pre-mRNA. The consensus sequences of the 5’ splice site is (C/A)AG ↓ GU(A/G)AGU (the splice site is denoted by a ↓), invariant nucleotides are underlined and of the 3’ splice site is (T/C)AG ↓ GU. These consensus sequences are well conserved within eukaryotic species from yeast to human (41). During the past several years, intron retention has been shown to be a facet of normal mRNA splicing, and intron-containing mRNA is associated with many cellular functions. For example, a fraction (0.1–20%) of bovine growth hormone cytosolic mRNA retains the last intron, intron D, in bovine anterior pituitary somatotrophs (6). An alternative mRNA of human nontransmembrane phosphotyrosine phosphatase (PTP-1B) retains the last intron and encodes a protein with a different C-terminal region. The amount of intron-retaining mRNA was increased upon growth factor stimulation (42). In some cases, such as mouse tyrosinase, intron retention serves as a negative regulator for either functional mRNA production (43) or functional protein synthesis as found for the kinase-deficient splice variants of Janus kinase 3 (44). Intron retention has also been shown to cause several types of genetic diseases; for example, the retention of intron 10 in the phosphofructokinase gene causes Tauri disease (45), and retention of intron 9 in CD44 causes certain cases of urinary bladder cancer (46).

Here, we show that human GRK1 has a splice variant,

3 K. Palczewski, X. Zhao, and M. Gelb, manuscript in preparation.

![Fig. 6. Relative abundance of GRK1a and GRK1b transcripts in human retina.](image)

Quantitative PCR was performed on four randomly selected donors. Upper panel, in each individual, the GRK1a level was set as 1.0. The length of the bars indicates the level of GRK1b message in comparison with GRK1a in each donor. Three independent results are shown as white, gray, and black bars. The inset is one representative experiment using two pairs of primers that amplify both GRK1a and GRK1b in the same reaction. Lower panel, another representative experiment using GCAP1 as an internal control in each PCR reaction.

![Fig. 7. In situ hybridization of GRK1b mRNA to human retina.](image)

Human retina was hybridized with digoxigenin-labeled antisense (panel A) and sense (panel B) human GRK1 intron 6 probes. The retinal layers are indicated as follows: OS, outer segments; IS, inner segments; GNL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; and NFL, nerve fiber layer. Bar = 50 μm. A specific hybridization of the inner segments of photoreceptors is observed with the antisense probe.
The splicing of GRK1 in both human and chicken suggests that the mammalian consensus sequences at only five out of nine positions. The 5' splice site consensus residues (Refs. 6 and 47), and intron inclusion (6). For example, intron retention has been obtained by RACE PCR and all three clones obtained by library screening with currently available antibodies. The de-
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