

Mouse Opsin

GENE STRUCTURE AND MOLECULAR BASIS OF MULTIPLE TRANSCRIPTS*

(Received for publication, March 16, 1990)

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The single copy mouse opsin gene produces five major transcripts, varying in size from 1.7 to 5.1 kilobases. The mRNAs are present at levels that vary over 2 orders of magnitude and can be detected as early as postnatal day 1. Each of the transcripts is polyadenylated and can be identified in polysome-bound RNA, suggesting that each is translated *in vivo*. To elucidate the molecular basis of this complex transcription pattern, we have characterized genomic fragments covering the entire mouse opsin gene, including several kilobases of 5'- and 3'-flanking regions. Transcription initiates at a single site 97 base pairs upstream of the translation start codon. Northern hybridization with exon- and intron-specific probes demonstrated that the various transcripts are not generated by partial or alternative splicing. Sequence analysis of the 3' end of the gene showed the presence of multiple polyadenylation signals. Analysis by polymerase chain reaction of the 3' end of opsin cDNA demonstrated that the complex transcription pattern originated from the selective use of these polyadenylation sites, generating transcripts that differ only in the length of the 3'-untranslated region. Transcript heterogeneity similar to that observed in mouse was also found in rat and, to a lesser degree, in human and frog opsin mRNAs.

Phototransduction in mammalian rod photoreceptors operates via a complex enzyme cascade initiated by the absorption of light by rhodopsin (1, 2). Rod rhodopsins are comprised of a chromophore, 11-*cis*-retinal, and a 40-kDa apoprotein, opsin (3, 4). The amino acid sequences of bovine (5, 6) and ovine opsins (7) consisting of 348 residues have been deter-

* This investigation was supported in part by National Eye Institute Grants EY08123 (to W. B.) and EY06085 and EY06639 (to J. F. M.); Individual National Research Service Award F32 EY06172-01 (to S. J. P.); grants (to W. B.) from the National Retinitis Pigmentosa Foundation/Fighting Blindness, Texas Advanced Technology Program, the Retina Research Foundation (Houston); a National Institutes of Health Biomedical Research Support Grant (to W. B.); and a grant from the Knights Templar Foundation (to M. R. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M55171.

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mined by peptide sequencing. The mammalian opsin genes that have been identified thus far are present in single copy in their respective genomes (8). The human gene was mapped to the long arm of chromosome 3 (9, 10), and the mouse gene, to chromosome 6 (11). Genes and cDNAs encoding opsins have been described for various vertebrate and invertebrate species, including bovine (12), human (13), *Drosophila* (14, 15), mouse (16), octopus (17), and chicken (18). The characteristic organization of the mammalian opsin gene is a 5-exon, 4-intron arrangement with highly homologous exon sequences and with precise conservation of intron positions. Mutations in the opsin gene may disrupt phototransduction and therefore lead to blindness. Recently, a point mutation in exon 1 (a C to A conversion in codon 23 resulting in a Pro to His substitution) of the human opsin gene has been implicated as causative of one form of autosomal dominant retinitis pigmentosa (19). In an earlier, unrelated study, an autosomal dominant retinitis pigmentosa gene in a large Irish pedigree was mapped to the long arm of chromosome 3 (20). In order to facilitate studies aimed at the establishment of a mouse model for this blinding disorder we have extended our preliminary characterization of the mouse opsin gene (16). It was shown previously that transcription of the gene produces at least five major transcripts (16, 21, 22). The sizes of the RNAs range from 1.7 to 5.1 kb,¹ and the various species are present in the rod cytoplasm at levels that vary over 2 orders of magnitude (16). The major transcript of the bovine opsin gene, in contrast, is a 3-kb species which was shown to contain a large 3'-untranslated region (12). The molecular basis leading to the heterogeneity observed in mouse is not understood.

In this paper, we present the complete sequence of the mouse opsin gene, including introns and flanking sequences (9.5 kb). We show that the transcript heterogeneity is not generated by alternative splicing or by the use of multiple initiation sites. The gene sequence contains several putative polyadenylation sites resembling the AATAAA consensus site (23) in the 3'-flanking region. We provide evidence, by PCR amplification of 3'-flanking sequences of opsin cDNA, that the transcripts observed are generated by the selective use of these polyadenylation sites. Moreover, we show that the pattern of multiple transcripts is not unique to the mouse, but is also present in the rat (four transcripts), human (three transcripts), and frog retina (two transcripts).

MATERIALS AND METHODS

RNA—Retinas of various vertebrate species (adult normal C57BL/6J mice, rats (Long Evans), Irish setter dogs, humans (a generous

¹ The abbreviations used are: kb, kilobase(s); PCR, polymerase chain reaction; PN, postnatal day; bp, base pair(s); mcs, multiple cloning site.

gift of the Lions' Eye Bank, Houston, TX), and frog (*Rana pipiens*) were excised from enucleated eyes and immediately dropped into liquid nitrogen. Total RNA was isolated according to Chirgwin *et al.* (24), and poly(A) mRNA was isolated by two sequential passages through oligo(dT) columns (25). In some cases, poly(A) mRNA was also isolated according to the Fastrack procedure (Invitrogen (26)). No apparent differences in mRNAs from these procedures were observed. For Northern blotting, RNA was electrophoretically fractionated on 1% agarose gels containing 2.2 M formaldehyde and transferred by capillary action (27) to Zetaprobe or Nytran membranes. Labeled opsin-specific oligonucleotides, opsin cDNA, or MOPS1 fragments were hybridized to the filters in 50% formamide containing either $6 \times$ SSC (0.90 M NaCl, 0.09 M sodium citrate) or $1.5 \times$ SSPE (0.36 M NaCl, 0.02 M sodium phosphate (pH 7.7), 0.002 M EDTA) at 42 °C. The filters were washed three times in $2 \times$, $0.5 \times$, and $0.1 \times$ SSC, 0.5% sodium dodecyl sulfate at 55 °C for 20 min each and exposed to Kodak XAR5 film with an intensifier screen at -70 °C for the times indicated in the figure legends. The transcription start point of opsin mRNA was determined by extension of synthetic antisense oligonucleotides according to Ingolia *et al.* (28).

Complementary DNA—The three cDNA clones M2–M4 (Fig. 1) were isolated as described previously (16, 29). Exon-specific fragments were isolated by digesting the 1.1-kb M2 insert with various restriction endonucleases according to the following strategy. M2 insert digested with *XhoI* yielded a 270-bp 5' fragment which is exon 1-specific (Fig. 1B, x1). The 848-bp 3' fragment was digested with *PvuII* to give a 339- and a 509-bp fragment. The 339-bp *XhoI/PvuII* fragment was digested with *RsaI*. The resulting 142-bp fragment is exon 3-specific (x3); the larger 197-bp fragment was then digested with *HaeIII* to yield a 102-bp exon 2 probe (x2). The 509-bp *PvuII/XhoI* fragment was digested with *SacI*, yielding two fragments that were digested with *MnII*. The resulting 194-bp fragment is exon 4-specific (x4), and the 140-bp fragment is exon 5-specific (Fig. 1B, x5). All exon-specific fragments were shown not to cross-hybridize to each other by a standard dot blot procedure and to hybridize to the original M2 cDNA (results not shown).

Genomic DNA—A 5-kb genomic *EcoRI* fragment (Fig. 1, MOPS1) was isolated as described previously (16). Genomic clones with approximately 15-kb inserts (λ MO1 and λ MO2) were isolated from a bacteriophage library which was prepared by cloning partial *MboI* digests into the *BamHI* site of EMBL3 (Clontech). *PstI* and *XbaI* subclones of MOPS1 and *EcoRI/SalI*, *XhoI*, and *BamHI* subclones representing flanking sequences of the opsin gene were generated using standard procedures (30) with pUC or Bluescript vectors. Subclones were sequenced using the double-stranded plasmid sequencing technique (31), M13 universal and sequence-specific primers, and Sequenase (U. S. Biochemical Corp.). Sequencing reactions followed the Sequenase protocol except that 100 ng of primer were used for each set, and incubations for annealing were omitted. The coding region and introns of opsin were sequenced on both strands (the untranslated regions in only one direction), using dITP, where necessary, to resolve compression problems. Radiolabeling of DNA fragments was by nick translation (30) or random priming (32).

Oligonucleotide Synthesis—Oligonucleotides W9 and W11–W13 were from Genetic Designs (Houston, TX), purified by preparative polyacrylamide gel electrophoresis (19% acrylamide, 1% bisacrylamide, 7.5 M urea), and allowed to diffuse at 4 °C into 500 μ l of H₂O overnight. An aliquot containing 100 ng of DNA was directly used for DNA sequencing. For PCR amplification, the eluate was concentrated by precipitation in 50% isopropyl alcohol. Oligonucleotides W34–W60 were synthesized on a PCR-MATE DNA synthesizer (model 391A, Applied Biosystems, Inc.) and deprotected in 14 M NH₄OH at 55–65 °C for 12–18 h. After lyophilization, the oligomers were dissolved in 300 μ l of water, quantitated by spectrophotometry ($1 A_{260\text{ nm}} = 33 \mu\text{g}$), and diluted to 100 ng/ μ l. End labeling of oligonucleotides was performed with T4 polynucleotide kinase and [γ -³²P] ATP.

Polymerase Chain Reaction—To map the polyadenylation sites in the 3' end of the opsin gene, the polymerase chain reaction (33) was modified to allow amplification of specific 3'-untranslated regions (34) of opsin cDNA. In the first step of this procedure, retinal cDNA was synthesized with reverse transcriptase using an oligonucleotide primer, 3' T₁₆AGCTATAGATCTAGACGCCGGCGTACGCC (3'-T₁₆-Clal/*EcoRV*/*BglIII*/*NotI*/*SphI* or 3'-T₁₆-mcs), consisting of an oligo(dT)₁₆ domain and a 29-nucleotide multiple cloning site (mcs). The conditions for cDNA synthesis were according to the manufacturer's protocol (Promega Biotech). In a second step, the cDNAs produced were used directly after a 10-fold dilution as a template for

PCR amplification. The cDNA regions to be amplified were delimited by a primer (5'-CCGCATGCGGCCGAGATCTAGA, mcs23) which contained the first 23 nucleotides of T₁₆-mcs, and by opsin-specific primers (W9–W59, Figs. 2 and 4). Thirty cycles of amplification were performed in a thermal cycler (Ericomp) at 92/72/55 °C (denaturation, 1 min; extension, 2 min; annealing, 2 min; final extension, 10 min) with 2.5–4 units of Taq polymerase (Perkin-Elmer Cetus Instruments) and $1 \times$ Taq buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 0.1% gelatin) modified to contain 0.9–1.5 mM MgCl₂. The optimal enzyme and Mg²⁺ (1–2 mM) concentrations were determined individually for each set of primers. The length of the amplified DNA fragments was determined after electrophoresis on 1% agarose gels, Southern blotting, and hybridization with opsin-specific probes. In some cases, fragments of interest were purified by agarose gel electrophoresis and sequenced directly.

RESULTS

Characterization of Mouse Opsin Clones—We previously described the isolation of a 5-kb genomic clone MOPS1 (16), which was shown to contain the complete opsin-coding sequence, including the four introns but only short flanking regions. In order to characterize larger genomic fragments containing several kilobases of 5'- and 3'-flanking sequences, we used MOPS1 to isolate two overlapping genomic EMBL3 clones (λ MO1 and λ MO2). λ MO1 is the parent clone for all genomic subclones (except MOPS1) described in Fig. 1A. It contains approximately 6 kb of sequence upstream from the translation start codon (ATG) and 3.5 kb of the 3'-untranslated region. The 1.3-kb *XhoI* fragment XX1, whose 3' *XhoI* site is located just 10 bp upstream from ATG, contains putative CCAAT and TATAA boxes (Fig. 2) and, presumably, other upstream transcription regulatory sequences. The 3'-flanking fragment ES4 contains several polyadenylation sites (see below). The cDNA clones M2–M4 (Fig. 1B) were described previously (16).

Structure and Sequence of the Mouse Opsin Gene—A complete composite sequence (9.5 kb) of the mouse opsin gene, starting with the 5' *XhoI* site of XX1 and ending at the 3'

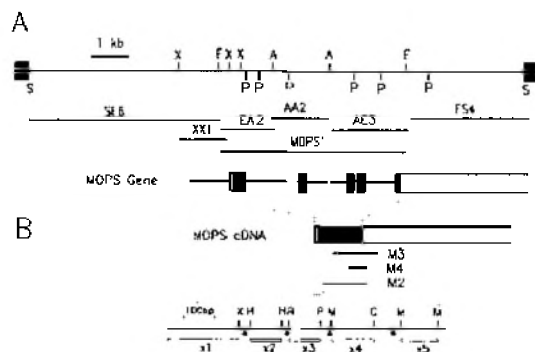


FIG. 1. Map of the mouse opsin gene and its mRNA. A, restriction map and extent of the mouse opsin genomic fragment λ MO1 (15 kb). The vector is indicated by hatched boxes. Single letters symbolize restriction sites that were used for sequencing and subcloning (S, *Sall* (λ); E, *EcoRI*; X, *XhoI*; A, *XbaI*; P, *PstI*). Lines underneath the restriction map represent the extent of genomic clones between two sites. Except for MOPS1, genomic clones are named using the single letter symbols of their flanking restriction sites, followed by the approximate length of the cloned insert in kilobases. Below MOPS1 is a schematic representation of the opsin gene. Boxes indicate the extent of exons and lines connecting the exons denote the length of introns. B, schematic representation of opsin cDNA. The filled box represents the coding sequence, and open boxes are the 5'- and 3'-untranslated regions. The actual 3' end of the structural gene is undetermined. The extent of cDNA clones M3, M4, and M2 is indicated below. A restriction map of M2 with sites used for excision of exon-specific fragments is shown at the bottom (M, *MnII*; X, *XhoI*; P, *PvuII*; H, *HaeIII*; R, *RsaI*; C, *SacI*). The positions of introns in M2 are marked with arrowheads. x1–x5, exon-specific probes.

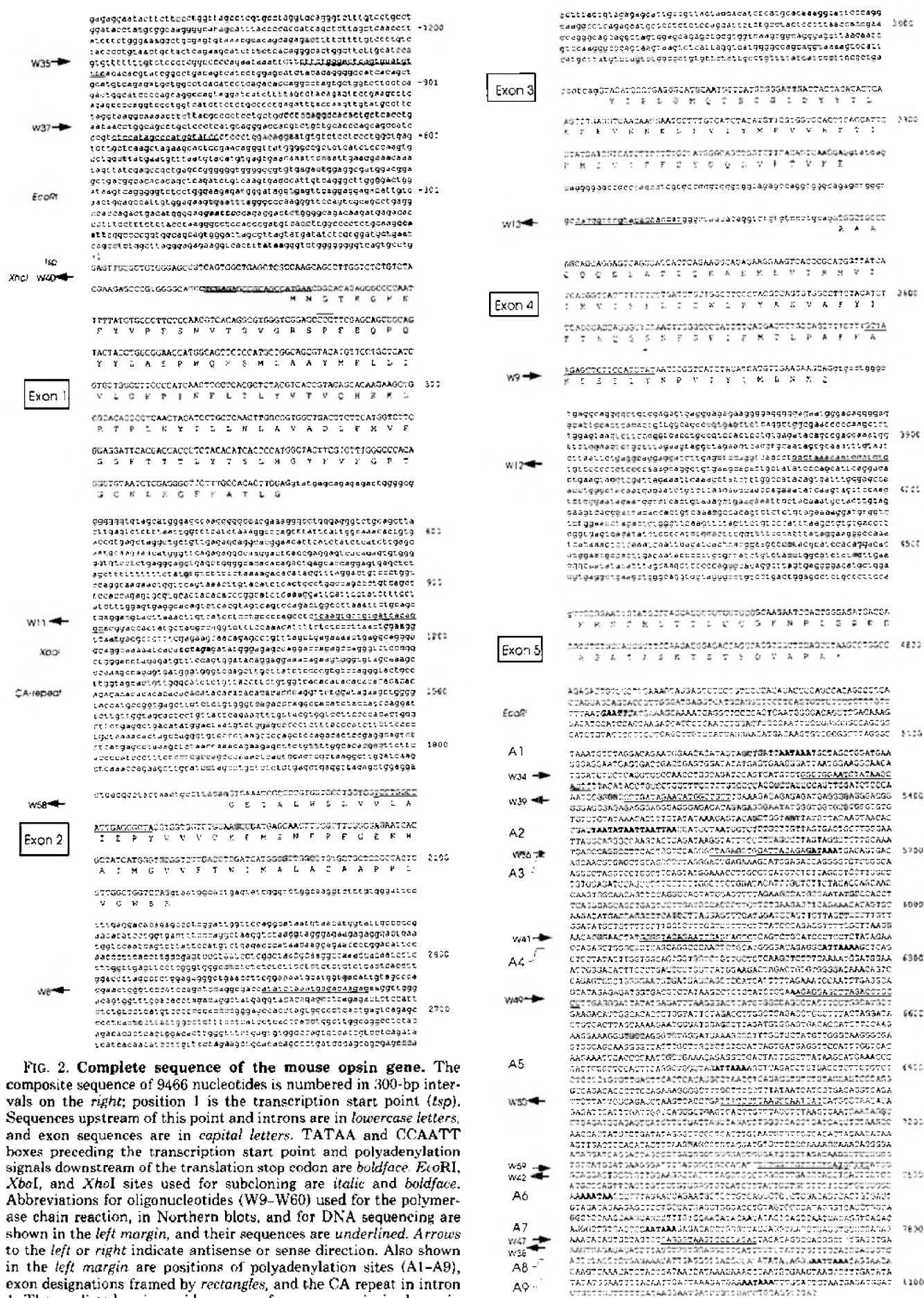


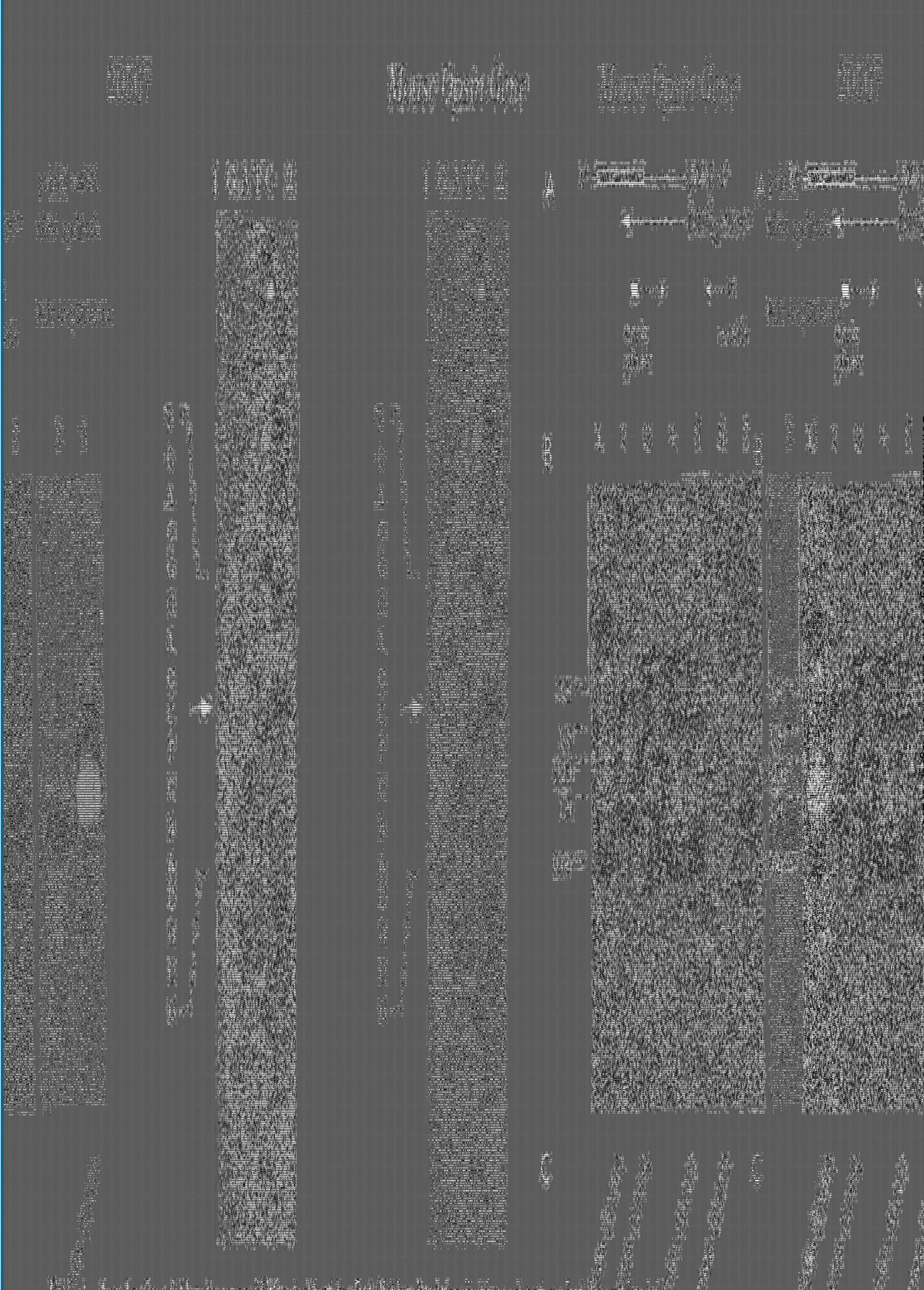
FIG. 2. Complete sequence of the mouse opsin gene. The composite sequence of 9466 nucleotides is numbered in 300-bp intervals on the right; position 1 is the transcription start point (*tsp*). Sequences upstream of this point and introns are in lowercase letters, and exon sequences are in capital letters. TATA and CCAAT boxes preceding the transcription start point and polyadenylation signals downstream of the translation stop codon are boldface. EcoRI, XbaI, and XhoI sites used for subcloning are italic and boldface. Abbreviations for oligonucleotides (W9–W60) used for the polymerase chain reaction, in Northern blots, and for DNA sequencing are shown in the left margin, and their sequences are underlined. Arrows to the left or right indicate antisense or sense direction. Also shown in the left margin are positions of polyadenylation sites (A1–A9), exon designations framed by rectangles, and the CA repeat in intron 1. The predicted amino acid sequence for mouse opsin is shown in single letter symbols underneath the exon sequences.

FIG. 2—continued

and of IS1, is shown in Fig. 2. The differences between these human and mouse sequences
 identified by identical sequence-based comparisons. The mouse sequences
 regions (6) within the same subregion and by comparison of each by comparison
 with the cDNA sequence of the M11. The results show that the mouse open gene
 that the mouse open gene (Exon 1) is 100% identical to the human open gene and
 human cDNA sequence. The mouse open gene (Exon 2) is 100% identical to the human
 open gene, and that the repeat region (Exon 3) is 100% identical to the human
 sequence. The mouse open gene (Exon 4) is 100% identical to the human open gene.
 open genes are highly conserved and diverge from each other. The mouse open gene
 containing 9 conserved regions across several different regions of the human
 The levels of the human and mouse genes were 1500, 1000, and 1000 units of human
 (1000, 1000, and 1000) units of human. The mouse open gene (Exon 1) is 100% identical
 (100%) sequence in percent identity. A comparison of the human and mouse sequences
 shown reveals that there is no sequence similarity between the human and mouse
 corresponding human and mouse genes. The mouse open gene (Exon 2) is 100% identical
 and the mouse open gene (Exon 3) is 100% identical to the human open gene.
 however, can be detected in human and mouse. The mouse open gene (Exon 4) is 100% identical
 to the human open gene. The mouse open gene (Exon 5) is 100% identical to the human
 of its junction with exon 1 (Fig. 2) in the corresponding human open gene. The mouse
 human open gene. The mouse open gene (Exon 6) is 100% identical to the human open gene.

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at the poly(A) tail are amplified using the polymerase chain reaction. The results show that, of nine putative polyadenylation sites in the 3'-untranslated region of the mouse opsin gene, five are utilized to produce the major species seen on the Northern blot. Use of the most proximal site thus produces the shortest transcript, whereas the most distal yields the longest. Many genes contain internal AATAAA sequences that apparently do not function in 3' processing, suggesting the requirement for additional signal sequences (23). A transcript heterogeneity similar to that in mouse is also observed in rat and, to a lesser extent, in human and frog (Fig. 4). No sequence information is available for the rat and frog opsin genes, and the 3' end of the bovine gene has been only partially sequenced (12). The human sequence (13) has at least two polyadenylation sites that may lead to differential polyadenylation. Apart from multiple poly(A) sites, the organization of the mouse opsin gene is relatively uncomplicated. With a single transcription starting point, no alternative splicing, and multiple polyadenylation sites, the mouse opsin gene is similar to a group of genes (46) encoding very diverse protein products, among them murine dihydrofolate reductase (48), chicken vimentin (49), and human N-ras (50). The human N-ras gene has two and the dihydrofolate reductase gene 11 functional polyadenylation sites. While 3'-untranslated sequences may play a role in regulation of gene expression or may affect RNA transport and stability (46), a functional significance of the multiple mouse opsin mRNA species remains elusive.

The recent discovery that a Pro to His mutation at position 23 near the N terminus of human opsin may be causative for one form of autosomal dominant retinitis pigmentosa (19), an inherited photoreceptor degeneration in the human retina, has renewed interest in the mouse opsin gene as a candidate gene to study retinal degeneration. Compared with the predicted human sequence (13), only 19 residues differ in mouse opsin (16). Pro²³ is present in the mouse sequence (Fig. 2) and is conserved in all sequenced vertebrate and invertebrate opsins and in related G protein receptors (51). Apart from asparagine-linked glycosylation, a precise function for the N-terminal end of opsin or for Pro²³ has not been determined. The detailed characterization of the mouse opsin gene and availability of cloned gene fragments as described in this study will allow the introduction of specific point mutations, the following of the consequences of expression of the mutated opsin gene in a transgenic mouse, and, possibly, the facilitation of the establishment of an animal model which mimics one autosomal dominant form of human retinitis pigmentosa.

Acknowledgments—We thank Drs. Gregory I. Liou and Richard Hurwitz for critically reading this paper. We are grateful to Dr. Kuyas Bugra for generating the exon-specific cDNA probes and to Nancy Baumann for help with the manuscript.

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