Dystrophin, a cytoskeletal membrane-associated protein,1 was first described as a 427-kDa protein encoded by the Duchenne muscular dystrophy (DMD) gene located on the X chromosome.2 It consists of four distinct domains: an N-terminal actin binding domain, a large spectrin-like rod domain, a cysteine-rich domain, and a unique carboxy-terminal domain that interacts with the dystrophin-associated proteins (DAPs).3 This late region of 420 amino acids is highly homologous to the dystrophin-related protein (DRP1 or utrophin), described by Love et al.,4 which is encoded by an autosomal DMD-like gene. Dystrophin is defective in the skeletal muscle of patients affected by Duchenne muscular dystrophy and in genetically dystrophic mice, mdx and mdx<sup>cv</sup>.5 It has been postulated that dystrophin, in association with cytoskeletal components and the DAP complex, affects muscle membrane stability and maintenance of ionic gradients.1

Dystrophin superfamily.1 Nonprogressive cognitive deficits are observed in DMD and patients with Becker muscular dystrophy (BMD).14-16

In mouse retina, three DMD gene products, full-length dystrophin, Dp260, and Dp71 were identified by D'Souza et al.10 Dp140, utrophin, G-utrophin and/or DRP2, highly homologous to Dp116 have been identified by Blake et al.12 and Roberts et al.13 In brain, the absence or the malfunction of dystrophin and/or C-terminal products of the DMD gene have been correlated with the nonprogressive cognitive deficits observed in DMD and patients with Becker muscular dystrophy (BMD).14-16

In mouse retina, three DMD gene products, full-length dystrophin, Dp260, and Dp71 were identified by D'Souza et al.10 Dp140, utrophin, G-utrophin and/or DRP2 were additionally identified in normal rat retina by Rodius et al.17 Moreover, patients with DMD gene mutations have an abnormal electoretinogram (ERG).18-20 It has been shown by Sigesmund et al.21 that 88% of DMD or BMD patients have reduced b-wave amplitudes under scotopic conditions, whereas the a-wave is unchanged. Patients with deletions in the central region of the gene have the most severe ERG changes.21,22 These studies and those of Pillers et al.23 that have shown that the mdx<sup>cv</sup> mouse,
with a mutation in intron 65 at the 3' end of the gene, had a dramatically reduced-amplitude b-wave, suggest that short DMD gene products may play a role in retinal neurotransmission. Stockton and Slaughter have shown that the b-wave reflects the ON-bipolar cell activity. Light-induced ON-bipolar cell depolarization causes an increase in extracellular potassium concentration in the outer plexiform layer (OPL). Subsequently, Müller glial cells, by transporting and releasing potassium in the vitreous humor, generate a potassium flux that corresponds to a positive polarity recorded as a b-wave in the ERG. DMD gene products may interact with members of the DAP complex and affect retinal transmission by altering membrane integrity, local ionic (i.e., K⁺) concentrations, or neurotransmitter (i.e., glutamate) concentrations.

Immunolabeling studies, with monoclonal antibodies prepared against the C-terminal domain common to all DMD gene products, localized dystrophin(s) in the OPL and more precisely, at the ultrastructural level, in presynaptic photoreceptor cells. Furthermore, in a recent study, Howard et al. with dystrophin isoform-specific antibodies were able to localize, in mouse retina, Dp260 in the OPL and Dp71, spliced for exon 78, in the inner limiting membrane. The purposes of the present work were to determine which of the dystrophin proteins, identified in the whole rat retina, were expressed in photoreceptor cells; and by using several antibodies prepared against diverse regions of dystrophins, to determine whether other cell types of the retina also express dystrophin proteins. To investigate the photoreceptor localization of dystrophin proteins, we examined the developmental pattern of the DMD gene products in the Royal College of Surgeons (RCS-rdy-p+) rat strain, described by Dowling and Sidman, that exhibits inherited progressive photoreceptor degeneration, resulting in total photoreceptor loss by 3 months of age. We also monitored the expression of DMD gene messengers by reverse transcription-polymerase chain reaction (RT-PCR) after mechanical fractionation of normal rat retina through the OPL.

We showed that Dp260 is exclusively expressed in photoreceptor cells thus indicating a presynaptic localization of this protein in the OPL. Furthermore, comparison of immunostaining with specific antibodies suggests that Dp71 spliced for the exon 78 may be expressed in Müller glial cells. These results provide a possible explanation for the role of these proteins in the b-wave generation.

**MATERIALS AND METHODS**

**Animals**

Twelve Wistar and RCS rats of each age (8, 15, 30, and 90 days) were used. Animals were deeply anesthetized by CO₂ inhalation and killed by cervical dislocation. All experiments were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Electroretinogram**

RCS black-eyed rats were bred from homozygous breeding pairs who had no detectable ERG result, confirming advanced retinal degeneration. Rats aged 15, 30, and 90 days were anesthetized with 6% sodium pentobarbital and placed in a Faraday's cage with a heating pad after overnight dark adaptation. Pupils were dilated with 0.5% tropicamide, and the cornea was anesthetized with 1% topical tetracaine. Electroretinograms were recorded with a cotton-wick electrode placed on the cornea and referenced to a needle electrode placed subcutaneously on the head. The ground electrode was placed on the tail. The upper and lower lids were retracted to hold open the eye. The recordings were amplified at gain of 10,000 and filtered with the low 0.1-Hz and high 1000-Hz cutoff filters from an amplifier (Universal Gould; Gould, France). Responses were digitized using a data acquisition board (Labmaster; Scientific Solutions, Solon, OH), connected to a personal computer. Experimental data were acquired and analyzed using the Patchit and Tack Software packages, respectively. Errors were expressed as SEM. The light for the stimulus was obtained from a 150-W xenon lamp bulb (Müller Instrument, Moosinning, Germany). The light beam was focused at infinity through a heat-absorbing filter and onto a hole in the Faraday's cage. The illumination intensity of the light stimulus was 2.9 cd/m² as measured with a luxmeter at the level of the rat eye. A computer-controlled shutter was used to deliver flashes of 100-msec duration.

**cDNA Synthesis**

Total RNA was extracted from rat retinas by the guanidium thiocyanate method established by Chomczynski and Sacchi. One microgram RNA was primed with random hexadeoxynucleotides and reverse transcribed using MuLV reverse transcriptase according to the recommendations of the manufacturer (Perkin Elmer, Norwalk, CT). To check the integrity and the concentration of the messengers, 1-µg/ml aliquots mRNAs were loaded on a 1% agarose gel.

**PCR Reactions**

PCR amplifications were performed on 1 µg cDNA with 200 µM of each dNTP, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl₂, 0.25 µM of each primer, and 2 U Taq polymerase (Extra-Pol; Eurobio, Les Ulis, France). Each cycle consisted of a heat-denaturation step at 94°C for 30 seconds, annealing of primers for 30 seconds, and polymerization at 72°C for 30 seconds. PCR conditions were adjusted so that the reactions were performed in the linear range of amplification, and β-actin was also amplified as a control for the amount of starting RNAs. We have previously shown that the expression level of β-actin remains constant in the rat developing retina and can be used as internal control. The sequences of the primers used for PCR, the number of cycles of amplification, and the predicted size of the amplified products are given in Table 1.

**Semi-quantification of the PCR Products**

An aliquot of each reaction was electrophoresed in 1X TAE buffer on 2% agarose gel. Gels were stained with ethidium bromide, and the PCR products were evaluated by measuring the fluorescence intensity of the bands with an image analyzer (TLC Scanner II; Camag, Muttenz, Switzerland). At each developmental stage, the relative amount of the target gene mRNA was calculated from the ratio of target gene product relative to β-actin gene product. Semi-quantification was realized on PCR products obtained from at least three reverse transcriptions. The ratios were shown in arbitrary units. Errors were expressed as SEM.

**Electrophoresis and Western Blot Analysis**

Rat retinas were homogenized at 4°C in 10 volumes (wt/vol) extraction buffer (0.32 M sucrose, 10 mM Tris-HCl, 1 mM
### Table 1. Oligonucleotides Used for PCR

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primers</th>
<th>Sequence (5' to 3')</th>
<th>Cycles</th>
<th>Product Size</th>
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<tr>
<td>Dp427</td>
<td>427.F</td>
<td>CTTCAGGAAGATGACAGAATCAG</td>
<td>35</td>
<td>314 bp</td>
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<td></td>
<td>427.R</td>
<td>TTGCTGAGAATGATTCTTCTGTA</td>
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<tr>
<td>Dp260</td>
<td>260.F</td>
<td>ATAGCGACAGCGATGAGTGGCT</td>
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<td>289 bp</td>
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<tr>
<td></td>
<td>260.R</td>
<td>TTCTTCTTCTCTCTAAACCT</td>
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<td>Dp140</td>
<td>140.F</td>
<td>GCATTGCCACTCTCTCTGAGC</td>
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<td>Dp71</td>
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<td>183 bp</td>
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<td></td>
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<tr>
<td>mGluR6</td>
<td>GI11R6.F</td>
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<td>GI11R6.R</td>
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<td>β-actin</td>
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<td>215 bp</td>
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<td></td>
<td>BA.R</td>
<td>TACAGCCCCGGTGGTTGAAA</td>
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<td>Rhodopsin</td>
<td>ROD.F</td>
<td>ATTCACACACCCACCTCCACC</td>
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<td>ROD.R</td>
<td>AGAGAGGCGACTCTCCAAGG</td>
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</table>

The choice of oligonucleotides was made according to the sequences available in GenBank; accession numbers are X03765 for β-actin, D13963 for mGluR6, and Z46957 for rhodopsin. Dystrophin primers correspond to the brain isoform (accession number X14182); 260.F, 140.F, and 71.F were chosen in the promoter sequences. The other dystrophin primers were designed in the mouse dystrophin mRNA sequence (accession number M68859).

EDTA, [pH 7.4]) containing a mixture of protease inhibitors and centrifuged at 1000g for 5 minutes. Supernatants were recovered and centrifuged at 17,000g for 30 minutes at 4°C. Pellets contained a crude membrane preparation and were recovered in the extraction buffer. Protein concentrations were determined using bovine serum albumin as a standard.

Protein extracts were resolved on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Twenty micrograms of proteins were loaded per well. Proteins were electrotransferred to nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). The efficiency of proteins transfer was controlled by both ponceau red staining of the blot and Coomassie blue staining of the remaining gel. Western blot analyses were blocked with 1% bovine serum albumin (BSA) and 3% dry milk (Bio-Rad, Hercules, CA) in phosphate-buffered saline (PBS) overnight at 4°C, then probed with primary antibodies diluted 1:100 in PBS and with horseradish peroxidase-labeled goat anti-mouse secondary antibody (Interchim, Montluçon, France) diluted 1:10,000 in PBS and 0.1% Tween 20.

Chemiluminescence detection was performed using a kit (Super Signal; Pierce, Evansville, IL) and documented on film (Hyperfilms; Amersham, Arlington Heights, IL).

### Antibodies

Five monoclonal antibodies were used in the characterization of the DMD gene products. These antibodies were directed against different domains of the full-length dystrophin (Table 2). 5G5, H5A3, and 5F3 antibodies have been produced and characterized in detail by Léger et al. and Fabbrizio et al. Dystrophin, H5A3, and DRP1 antibodies were from Novocastra (Newcastle-on-Tyne, UK). Anti-vimentin antibody 2A1 (Sigma-Alrich, Saint-Quentin, France) was also used in this study to identify glial cells.

### Immunocytochemistry

Rat eyes were removed and frozen in liquid nitrogen using a cryoprotector (Cryomatrix, Life Sciences, UK). Cryostat sections, 7-μm thick, were achieved to obtain vertical sections of the retina that were collected on gelatin-coated glass slides.

### Table 2. Characteristics of the Dystrophin Monoclonal Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Full-length Dystrophin</th>
<th>Amino Acid Position of the Antigen</th>
<th>Exons in DMD Gene*</th>
<th>Specificity</th>
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</thead>
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<tr>
<td>Dys1</td>
<td>1181-1388</td>
<td>Exons 26-30</td>
<td>Dystrophin</td>
<td></td>
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<tr>
<td>5G5</td>
<td>1173-1728</td>
<td>Exons 26-37</td>
<td>Dystrophin, Dp260</td>
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<tr>
<td>H5A3</td>
<td>3357-3660</td>
<td>Exons 71-74</td>
<td>Dystrophin, utrophin, Dp260, Dp140, Dp116, Dp71, G-utrophin, DRP2</td>
<td></td>
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<tr>
<td>Dys2</td>
<td>3669-3685</td>
<td>78</td>
<td>Dystrophin, Dp260, Dp140, Dp116, Dp71</td>
<td></td>
</tr>
<tr>
<td>5F3</td>
<td>Alternative 31 amino acids</td>
<td>Terminal end (founder sequence)</td>
<td>79</td>
<td>Dp71†</td>
</tr>
</tbody>
</table>

* Exon boundaries are taken from Roberts et al.
† Dp71 founder sequence.
FIGURE 1. Electroretinogram study of RCS-rdy-p+ and Wistar rats during postnatal development showing a-wave amplitude evolution (in microvolts) from 15 to 90 days of age. Data of RCS-rdy-p+ are presented as mean ± SEM (n = 4). Those of Wistar control are representative of three experiments.

Immunocytochemical labeling was performed using an indirect fluorescence method. After 5 minutes of permeabilization with 0.1% Triton X-100 in PBS and blocking in PBS (0.01 M, pH 7.4) containing 0.1% BSA, sections were incubated with primary antibodies, diluted 1:100 for 2A1 or 1:10 for all the others. Fixation in 4% paraformaldehyde was performed during this step because of the sensitivity of the anti-dystrophin antibodies. Goat anti-mouse secondary antibodies (Interchim, France) were used coupled to either bodipy or Texas red and diluted 1:100 in PBS containing 0.1% Tween-20. Sections were mounted in 50% glycerol in PBS and examined with a photomicroscope (Optiphot 2; Nikon, Tokyo, Japan) using a ×40 objective.

Retinal Horizontal Sections
After dissection of the retina, the photoreceptor layer was isolated from the inner part of the retina by horizontal sectioning through the OPL with a vibratome, as described previously.35

RESULTS
Postnatal Development of ERG in RCS Rats
As a first step, we examined in our experimental conditions the decrease in ERG amplitudes in RCS rats, which have an inherited photoreceptor degeneration. Figure 1 shows the decline of the ERG a-wave amplitude during postnatal development of the RCS rat (15, 30, and 90 days) compared with the Wistar rat. At 15 days, the a-wave amplitude was higher in RCS than in Wistar rats, this result was in accordance with previous observations.34,43 In RCS rats, the a-wave, which reflects photoreceptor activity, decreased to a minimum value below 20 μV at 3 months of age; thus, indicating an almost complete degeneration of photoreceptors, as reported by Kaitz and Auerbach.43 This provided us with a model to study the expression of the DMD gene products after photoreceptor disappearance.

Dystrophins mRNAs Pattern in Developing RCS Rat Retina
To investigate the distribution of DMD gene products in retina of RCS rats, we examined the expression patterns of full-length dystrophin, Dp260, Dp140, and Dp71 at different developmental stages of the RCS rat (days 8, 15, 30, and 90). Dystrophin...
mRNAs were analyzed by semiquantitative RT-PCR (Fig. 2), and rhodopsin mRNA levels of expression were used as a further control of the degeneration process of photoreceptors. The amount of this later mRNA increased until day 15 (Fig. 2, rhodopsin, lanes 1 and 2) and decreased thereafter (lane 3). At 90 days after birth (lane 4), this mRNA disappeared completely. Comparison of the progressive decrease of rhodopsin mRNA and the disappearance of the ERG (Fig. 1) indicated that synaptic transmission perturbation preceded photoreceptor disappearance. In these experimental conditions, we observed that all dystrophin mRNAs were present at 8 days of postnatal development (lane 1). For full-length dystrophin and Dp71, no significant variations of the mRNA were observed with further development. Their relative expression patterns were almost constant from 8 to 30 days of development (lanes 1 and 2) and rose slightly at day 90 (lane 3). The amount of Dp140 messenger increased progressively during development, the mRNA level at 90 days (lane 4) being roughly twice as high as that at day 8 (lane 1). The developmental patterns for these dystrophin transcripts were comparable to those reported in the Wistar albino rats. In contrast, Dp260 mRNA increased until day 15 (lane 2), remained constant until day 30 (lane 3), but dramatically decreased at day 90 (lane 4), whereas it was still expressed in adult Wistar rat retina. Thus, Dp260 messenger expression decreased concomitantly with photoreceptor degeneration, whereas all the other transcripts of the DMD gene remained, even after photoreceptor disappearance.

Dystrophins Proteins Pattern in Developing RCS Rat Retina

At the protein level, we examined retinal homogenates from RCS rats using the monoclonal antibody HS3A (Fig. 4A). Five protein bands were revealed by this antibody throughout postnatal development. Bands with an apparent molecular mass of 400 kDa and 116 kDa most likely corresponded to dystrophin or utrophin and to Dp116, G-utrophin, or DRP2, respectively.
These bands showed a progressive increase between 8 and 90 days (lanes 1 through 4). The expression of Dp260, Dp140, and Dp71 increased progressively from 8 to 30 days (lanes 1 through 3); however, at 90 days (lane 4), Dp260 became undetectable, Dp140 slightly decreased, and Dp71 markedly increased during the period analyzed. These results were confirmed using the Dys2 antibody (Fig. 4B). This antibody, prepared against the C-terminal-specific domain of dystrophin, recognized two bands at 140 kDa and 71 kDa in the 90-day-old RCS rat retina. No band was observed around 116 kDa with Dys2, suggesting that the band revealed with H5A3 may correspond to G-utrophin, described in the brain by Blake et al.,12 or to DRP2 described in the mouse eye by Dixon et al.45 Overexposure of the same blot revealed, with an unavoidable background increase, an additional band, with an apparent molecular mass of 400 kDa (data not shown). However, even after such a long exposure, we were not able to detect a band at 260 kDa. This was in contrast with previous developmental study that showed that, in Wistar rats, Dp260 was still expressed in adult retina.17 Control experiments were performed to show that the disappearance of Dp260 was not caused by altered subcellular fractionation behavior of this DMD gene product (data not shown). Altogether, these results indicate that expression of Dp260 is related to the presence of intact photoreceptor neurons.

**Figure 5.** Immunohistochemical analysis of retinas from RCS-rdy-p+ and Wistar rats using H5A3 antibody. Section from RCS-rdy-p+ rat at postnatal days 8 (A), 15 (B), 30 (C), and 90 (D), compared with a section from a 90-day-old Wistar rat (E). Punctate signal increased progressively in the OPL from 8 to 30 days (A, B, C); blood vessels were stained at 15 days (B, asterisk) and the GCL at 30 days (C). In adult rats (D), immunostaining revealed dystrophin expression in thick-stemmed processes, whereas the control Wistar rat at 90 days (E) still exhibited a stain in the OPL (punctate), in the GCL, and around blood vessels. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar, 20 μm (A).

**Distribution of Dystrophins in Developing RCS Rat Retina**

To investigate whether intact retinal organization was required for the specialized dystrophin distribution seen in the adult Wistar rat with Dys2 antibody,17 we stained cryostat sections at different ages with the H5A3 antibody, which recognized five DMD and utrophin gene products (Fig. 5). In the 8-day-old retina, immunoreactivity was weak and punctate distributed in the OPL (Fig. 5A). This immunostaining pattern was more prominent and punctate in 15-day-old RCS rat retina (Fig. 5B). Furthermore, at this stage retinal blood vessels were also stained (asterisk in Fig. 5B, 5C, 5E). After 30 days, retinal degeneration of photoreceptors commences, but dystrophins and/or utrophins continued to be seen as punctate immunoreactivity restricted to the OPL (Fig. 5C). At this stage, immunoreactivity appeared in the ganglion cell layer (GCL), comparable with that observed in the Wistar adult rat (Fig. 5E). In the 90-day-old retina (Fig. 5D), most photoreceptors had degenerated and formed a broad cell debris zone in the outer retina in which neither the outer nuclear nor the OPL could be discriminated any more. Dystrophins immunoreactivity, however, was still present, but not in a punctate pattern. In contrast, the GCL appeared heavily stained, and immunoreactive fibers extended into the inner plexiform and inner nuclear layers, a pattern that was not observed in Wistar rats of the same age (Fig. 5E). Therefore, immunostaining analysis indicated that inner retinal cells kept the ability to express dystrophins in the absence of photoreceptors, but the dystrophins distribution was altered when the retinal cell organization was disrupted.

**Cellular Localization of DMD Gene Products and Utrophin in Normal and RCS Rat Retinae**

In the absence of specific antibodies against each member of the dystrophin superfamily, and to analyze further their cellular localization, immunostaining analyses of adult Wistar and RCS rats were performed with several antibodies that were generated against specific regions of the dystrophin sequence (Table 2). Antibodies were characterized by western blot of retinal extracts from adult Wistar rats (Fig. 6) before their use for immunostaining analyses. As previously shown,17 H5A3 recognized five bands that corresponded to full-length dystrophin.
ONL

FIGURE 7. Immunohistochemical analysis of retinas from RCS-rdy-p+ (B, D, F, H, J, K) and Wistar rats (A, C, E, G, I), using several antibodies against the dystrophin superfamily. Sections from 90-day-old rats were immunostained with the following antibodies: Dys1 (A, B), 5G5 (C, D), Dys2 (E, F), 5F3 (G, H), DRP1 (I, J), and 2A1 (K). An autofluorescent debris zone (DZ, B, J) takes the place of the ONL in RCS-rdy-p+ rats. In Wistar rats only the Dys2 antibody (E) stained blood vessels (*asterisk*). In RCS-rdy-p+ rats only 5F3 (H) and DRP1 (J) antibodies labeled thick stem processes. Staining with the 2A1 antibody (K), which exclusively labeled the glial cells, suggested that Dp71f and utrophin are expressed in Müller glial cells. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar, 20 μm.

and/or utrophin, Dp260, Dp140, G-utrophin and/or DRP2, and Dp71 (Fig. 6A). The Dys2 antibody generated against the C-terminal-specific domain of dystrophin revealed four bands that correspond to full-length dystrophin and three shorter products of the DMD gene: Dp260, Dp140, and Dp71 (Fig. 6B). This antibody did not cross-react with utrophin. The 5G5 monoclonal antibody raised against the central part of dystrophin detected two bands that corresponded to full-length dystrophin and Dp260 (Fig. 6C). The Dys1 (full-length dystrophin-specific) and DRP1 (utrophin-specific) antibodies detected bands at 400 kDa (Figs. 6D, 6E). Finally, 5F3, which has been produced against the last 31 residues (founder sequence) of Dp71 spliced for exon 78 revealed a single band (Fig. 6F) corresponding to Dp71f (f denotes founder sequence). Because of the splicing of exon 78, the Dp71f cannot be detected with the Dys2 antibody.

Subsequently, we compared a series of retinal sections from 90-day-old Wistar and RCS rats immunostained with these antibodies (Fig. 7). Full-length dystrophin, revealed by Dys1 antibody was concentrated as a punctate staining in the OPL in normal rats (Fig. 7A), whereas the same staining was undetectable in retinal sections from RCS rats (Fig. 7B), suggesting that full-length dystrophin was restricted to the OPL. The labeling seen in the debris zone of RCS rats, also observed in sections stained only with the secondary antibody, was caused by the autofluorescence of the photoreceptor products (DZ in Figs. 7B, 7J). When immunostaining was performed with the 5G5 antibody (full-length dystrophin and Dp260-specific; Figs. 7C, 7D), the labeling pattern was very similar to that observed with the Dys1 antibody, indicating that Dp260 is also confined to the OPL in normal rats. Using Dys2, the OPL was stained in normal rats whereas, as observed for Dys1 and 5G5 antibodies, staining in RCS rat retina was undetectable (Figs. 7E, 7F). This absence of labeling most likely accounted for a diffusion of the immunoreactivity as a consequence of the known degenerative changes induced in the inner retina by the photoreceptor cell degeneration. In addition, with the Dys2 antibody, strong labeling of retinal blood vessels was observed in normal rats (Fig. 7E, asterisk), suggesting expression of Dp71 and/or Dp140 around blood vessels. The absence of labeling with these antibodies in RCS rat retinal blood vessels may be caused by profound changes in the vascular system of this rat.

Dp71f in normal rat retinae, revealed with the 5F3 antibody, showed a concentration of immunoreactivity in the GCL (Fig. 7G). In contrast to the dotted pattern observed in the OPL for dystrophin and Dp260, the GCL immunostaining of Dp71f seemed to be more concentrated in cell bodies at the border between retina and vitreous humor. In retinae from RCS rats immunoreactivity was intensified in the GCL, and thick-stemmed processes appeared heavily labeled by this antibody (Fig. 7H). Similar to Dp71f, utrophin was localized in the GCL of the normal retina (Fig. 7I) and in the thick processes of the RCS retina (Fig. 7J). To try to identify the origin of these thick processes, we stained retinae from RCS with a vimentin antibody (Fig. 7K), a specific marker currently used to identify Müller glial cells. Indeed, works of Roque and Caldwell46 have shown similar structures in RCS retinae that expressed vimentin and that were therefore identified as Müller glial cell processes. It is clear that the stain obtained with anti-vimentin antibody (Fig. 7K) is similar to the one observed with 5F3 (Fig. 7H) and DRP1 (Fig. 7I) antibodies, thus suggesting a Müller glial cell localization of Dp71f and utrophin.

A comparison by western blot analysis and immunocytochemistry of the different staining patterns obtained with these
antibodies allowed us to identify and localize the different DMD gene products in the normal and degenerated rat retina.

**DISCUSSION**

Here, we approached the distribution of the DMD gene products between the outer and the inner rat retina by analyzing their postnatal expression pattern in rat retinas that exhibit progressive photoreceptor disappearance and after mechanical separation of photoreceptor cell layer of Wistar rats. Moreover, to specify the cellular localization of these proteins, we compared the immunostains of retina slices with antibodies that were either specific for a single protein (Dysl, DRP1, and 5F3) or for several (H5A3, Dys2, and 5G5). By this approach we circumvented the lack of specific antibodies raised against each member of the dystrophin superfamily. We provided evidence that Dp260 is localized in photoreceptor cells but that shorter DMD gene products (i.e., Dp71f) are expressed in glial cells.

**Dp260 Localization in Photoreceptor Cells**

Dp260 disappearance paralleled photoreceptor disappearance in RCS rats. Consistent with this, we found that in Wistar rats with the 5G5 antibody, which recognized full-length dystrophin and Dp260, only a punctate stain was seen in the OPL. In accordance with our results Kameya et al.\(^{33}\) and Howard et al.\(^{33}\) obtained the same stain in the OPL of the malx mouse retina, a strain deficient in dystrophin. In addition, analysis of retinal slices confirmed that the Dp260 mRNA was exclusively expressed in the photoreceptor cell layer. Thus, on the basis of these results we suggest a presynaptic localization of Dp260. Furthermore, because the rat retina is rod dominant (97% photoreceptors), this indicates that Dp260 in this species is mainly expressed in rods.

**Full-Length Dystrophin, Dp140, and Dp71 Localization**

In normal rat retina, the Dys1 antibody (full-length dystrophin-specific) stained only the OPL. Nonetheless, because by mechanical separation we found that this dystrophin transcript was expressed in both the outer and the inner retina, we cannot exclude the possibility that full-length dystrophin could be expressed in photoreceptors and also in secondary neurons (i.e., bipolar or horizontal cells). The absence of immunoreactivity in adult RCS rats could be explained by the profound changes that occur in the architecture of the RCS rat retina. These could induce a diffusion of the stain that became undetectable or was masked by the large autofluorescent debris zone.\(^{46}\)

Because of its epitope localization, the Dys2 antibody detects dystrophin, Dp260, Dp140, and Dp71 (not spliced for exon 78), but is unable to detect Dp71f. This antibody stained the OPL and blood vessels. Because Dys1 and 5G5 antibodies did not stain blood vessels, we propose that the shorter DMD gene products Dp140 and/or Dp71 are expressed in one of the glial components that surround retinal vessels. In the absence of antibodies that recognize Dp140 or Dp71 not spliced for exon 78 exclusively, the latter view is supported by recent findings showing that Dp140 is expressed in one of the glial components along blood vessels in mouse cerebral cortex\(^{36}\) and that Dp71 is expressed in different glial cell lines.\(^{48}\) The absence of staining in the blood vessels of adult RCS rats could be a consequence of the vascular modifications that occur concomitantly with photoreceptor degeneration.\(^{49}\) In addition, Dp140 mRNA and protein had a different evolution during the postnatal development of the RCS rat (Figs. 2, 4), suggesting a posttranscriptional mechanism of regulation. This type of mechanism has been described for the RI subunit of cyclic adenosine monophosphate (cAMP)-dependent protein kinase in retina.\(^{50}\)

**Dp71f Localization in Müller Glial Cells**

To date, no studies designed to establish the cellular localization of the Dp71 protein within the retina have been reported. Howard et al.\(^{33}\) proposed that Dp71 spliced for exon 78 was localized in the inner limiting membrane. Here, we showed that two isoforms of Dp71 may have a different cellular localization. The immunostaining of Dp71 spliced for exon 78 differed greatly from that of the nonspliced isoform. Thus the 5F3 antibody, specific for Dp71f, clearly stained the GCL, and no immunoreactivity was observed in the OPL or around the blood vessels. This point was in disagreement with observations of Howard et al.\(^{53}\) who detected Dp71f around vascular elements. This apparent discrepancy may be because of differences in antibody epitopes or in species studied. In RCS rats, the staining of thick-stemmed process obtained with 5F3 was similar to the one observed with an anti-vimentin antibody stain that specifically labels Müller glial cells.\(^{46}\) Differences in the staining pattern for Dp71f between control and RCS rats could be explained by the enhanced expression of this protein. Indeed, an increase of proteins, normally expressed at low levels in Müller glial cells is thought to be a general phenomenon in the RCS rat.\(^{46}\) In addition, the enrichment of Dp71 mRNA in the inner retina (Fig. 3) led us to propose a Müller glial cell localization of Dp71f in normal rats, although we cannot fully rule out the possibility that this protein is localized in a different cell type in normal rat retina. Furthermore, in cultured Müller glial cells from dissociated rat retina, we were able to detect with the 5F3 antibody the expression of Dp71f (Claudepierre T, Rendon A., unpublished results). At present, the function of alternative splicing in Dp71 is unknown. Experiments performed with transgenic mice have shown that Dp71, spliced or not for exon 78, cannot replace the function of full-length dystrophin, although it restores the DAP complex.\(^{51, 52}\) Moreover, Kramarcy et al.\(^{53}\) and Austin et al.\(^{11}\) have shown that multiple isoforms of Dp71 have a cell- and a tissue-specific expression.

**Utrophin Localization in Müller Glial Cells**

Utrophin labeling was similar to that of Dp71f and led us to propose that Müller glial cells express both utrophin and Dp71f. In cultured Müller glial cells, we detected utrophin (TC and AR, unpublished results). In accordance with our results showing glial localization, utrophin is known to be enriched in the foot processes of astrocytes.\(^{54}\) Our study discloses two points about previous works in rat\(^{55}\) and mouse retinae.\(^{27}\) In rat, utrophin has been observed, with antibodies different from the one used here, to be situated in ganglion or amacrine cells but not in the OPL.\(^{25}\) Whereas in mouse, utrophin immunostaining is only localized in the OPL and around blood vessels but not in the GCL.\(^{27}\) Different antibody reactivity and/or species differences may be the cause of these contradictory

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observations. Further studies are needed to explore the role of utrophin in the retina.

Implication of DMD Gene Products in b-Wave Generation

In animal models, absence of full-length dystrophin in the mdx mouse does not induce a perturbation of the b-wave.\(^19\)\(^\text{19}\) In mdx\(^\text{cv}\) mice,\(^3\) in which expression of all DMD gene products is disrupted, a delayed implicit time and a reduced amplitude in the b-wave have been characterized.\(^23\) It has been suggested that the abnormal visual phenotype was caused primarily by the absence of the Dp260 isoform.\(^10\) To clarify the role of Dp260, Araki et al.\(^56\) have produced exon 52 knockout mice, in which expression of Dp260 and Dp140 was impaired. In these mice, the b-wave shows a normal amplitude, thus implying that neither Dp260 nor Dp140 are directly responsible for the b-wave generation. This mouse strain displays increased implicit time,\(^47\) suggesting the implication of Dp260 or Dp140 in this mechanism. In the present study, we provide evidence for a presynaptic localization in the OPL of Dp260 in the photoreceptor cells. This is in agreement with recent reports that localize β-dystroglycan in the OPL in photoreceptor cells.\(^57\)\(^-\)\(^59\) Interestingly, the exon 52 knockout mice show an absence of β-dystroglycan from the OPL, whereas the mdx mouse, which does not express full-length dystrophin, has a normal localization of β-dystroglycan.\(^47\) Thus, together with our findings, it is possible to suggest that Dp260 would be responsible for the localization of the DAP complex within the photoreceptor terminals. It has been hypothesized that the dystrophin–DAP complex in the OPL is responsible for the localization of the N-type voltage-gated calcium channel\(^57\) or glutamate transporters.\(^32\) Because exon 52 knockout mice show no significant changes in the amplitude of the b-wave, it has been pointed out by Kameya et al.\(^27\) that Dp71 may play an important role in generation of the b-wave in the ERG. Because Müller glial cells are involved in the formation of the b-wave, the observation that Dp71 spliced for exon 78 is localized in these glial cells (Fig. 7H) is consistent with the possible involvement of this Dp71 isoform in b-wave generation. Interestingly, β-dystroglycan is present in the endfoot of the Müller cells.\(^57\)\(^-\)\(^59\) At this localization, the Dp71f-DAP complex in Müller glial cells may be involved in b-wave genesis, by controlling \(K^+\) release in the vitreous humor.

In conclusion, although the function of the dystrophin–DAP complex in retina is not yet clear, the fact that Dp260 appears to be localized in photoreceptors, in particular in the presynaptic site, and that Dp71f is localized in Müller glial cells supports the idea that the abnormal ERG observed in patients with DMD and in mdx\(^\text{cv}\) mice is not caused by the alteration of a single product of the DMD gene but may result from functional disturbances in different types of cells that contribute to the generation of the b-wave and that express various dystrophins.

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References

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