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Identification and Characterization of a Neuroretina-Specific Enhancer Element in the Quail *Pax-6* (*Pax-QNR*) Gene

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Using nuclear run-on assays, we showed that the tissue-specific expression of quail *Pax-6* (*Pax-QNR*) P0-initiated mRNAs is due in part to regulation of the gene at the transcriptional level. Regulatory sequences governing neuroretina-specific expression of the P0-initiated mRNAs were investigated. By using reporter-based expression assays, we characterized a region within the *Pax-QNR* gene, located 7.5 kbp downstream from the P0 promoter, that functions as an enhancer in neuroretina cells but not in nonexpressing P0-initiated mRNA cells (quail embryo cells and quail retinal pigment epithelial cells). This enhancer element functioned in a position- and orientation-independent manner both on the *Pax-QNR* P0 promoter and the heterologous thymidine kinase promoter. Moreover, this enhancer element exhibited a developmental stage-specific activity during embryonic neuroretina development: in contrast to activity at day E7, the enhancer activity was very weak at day E5. This paralleled the level of expression of P0-initiated mRNAs observed at the same stages. Using footprinting, gel retardation, and Southwestern (DNA-protein) analysis, we demonstrated the existence of four neuroretina-specific nuclear protein-binding sites, involving multiple unknown factors. In addition we showed that the quail enhancer element is structurally and functionally conserved in mice. All of these results strongly suggest that this enhancer element may contribute to the neuroretina-specific transcriptional regulation of the *Pax-6* gene in vivo.

A large number of vertebrate developmental control genes have been identified by their homology to *Drosophila* genes that regulate pattern formation and contain conserved DNA binding domains, like the homeobox or the paired box (for a review, see reference 14). The *Pax* family, identified in the vertebrate genome by their homology to the paired box sequences of the *Drosophila* segmentation gene *paired*, consists of nine unlinked genes (36, 44) highly conserved during evolution, in various distantly related species such as nematodes, zebra fish, *Drosophila* species, mice, humans, quails, and chickens (25, 27, 33; for a review, see reference 8). The paired box is present alone (in the genes *Pax-1*, *Pax-2*, *Pax-5*, and *Pax-8*) or together with the homeobox domain (*Pax-3*, *Pax-4*, *Pax-6*, and *Pax-7*) (for a review, see reference 41). The predicted structure, nuclear localization, and in vitro binding strongly indicate that Pax proteins are transcriptional activators, and this has been directly demonstrated for the Pax-1 (7), Pax-2 (16), Pax-5 (1), Pax-6 (31), and Pax-8 (46) proteins.

The *Pax* genes are expressed in a spatially and temporally restricted pattern during embryonic development (for a review, see reference 41), indicating that these genes may be involved in the control of morphogenesis and pattern formation. Indeed, the importance of these genes in development is demonstrated by the developmental alterations in mice associated with mutations in the *Pax* genes (for a review, see reference 41). Mutations in the *Pax-6* gene have been associated with the mouse mutation *small eye* (21), and the corresponding human gene (*AN*) has been found deleted or mutated in many cases of the human disorder aniridia (17; for a review, see reference 38). Moreover, this gene is the homolog of the *eyeless* gene of *Drosophila* species, which is also involved in eye morphogenesis (33). The *Pax-6* gene is expressed in the developing central nervous system, the optic cup, the lens, and the overlying epithelium prior to morphological differentiation, and later in the neuronal layer of the retina (25, 27, 43). All of this strongly suggests that *Pax-6* may be involved in the regulation of some of the inductive events that occur during the formation of the eye. In addition, *Pax-6* is also expressed in the endocrine pancreas, where the cells have some traits in common with neurons, despite their distinct embryological origin (42). Thus, the restricted expression of this gene suggests transcriptional regulation of expression, with sequence-specific DNA-binding proteins interacting with tissue-specific enhancer and promoter elements. However, the *cis*-acting elements and *trans*-acting factors for directing the tissue-specific expression of the *Pax-6* gene have not yet been identified.

Our research focuses on the regulatory elements involved in the tissue-specific expression of the quail *Pax-6* gene (termed *Pax-QNR*). This gene expresses at least two mRNAs, named MC29-QNR2 and B1, differing by their 5′ untranslated region and resulting from alternative splicing (13). We have recently characterized the promoter region initiating the MC29-QNR2 mRNA (termed P0), and we have shown that the promoter sequences located between positions −1386 and +127 (with respect to the transcription start site) function in *Pax-QNR*-expressing cells (quail neuroretina cells [QNR]) and *Pax-QNR*-nonexpressing cells (quail embryo cells [QEC]) (31). This finding suggested that tissue-specific expression of *Pax-QNR* requires additional upstream or downstream unidentified sequences or is the result of posttranscriptional regulation, as for the N-*myc* gene (2).

In this report, we show that neuroretina-specific expression of P0-initiated mRNAs is, in part, the result of transcriptional control. We have analyzed further the molecular mechanisms responsible for this neuroretina-specific regulation. We have identified a neuroretina-specific 3′ enhancer in the quail *Pax-6* gene, located 7.5 kbp downstream from the P0 promoter. In

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constructs containing this promoter, the 3′ enhancer functions in a cell type-specific manner, increasing the activity of the reporter gene from the P0 or the minimal thymidine kinase (TK) promoter in QNR, but not in cells in which the P0-initiated mRNAs are not detected. We have shown that this neuroretina-specific enhancer functions in a developmental stage-dependent manner, and we have demonstrated the existence of nuclear proteins in neuroretina cells that can interact specifically with this enhancer region. In addition, this enhancer is also structurally and functionally conserved in the mouse *Pax-6* gene, strongly suggesting that this regulatory element plays an important role in *Pax-6* gene expression in the neuroretina.

MATERIALS AND METHODS

Oligonucleotides. The following set of synthetic oligonucleotides was used (all sequences are written 5′ to 3′): 1F, gatcCTATTTGTCGTGACGATGCAATTTTCA; 1R, gatcTGAAAATTGCATCGTCACGACAAATAG; 2F, gatcCTGTCATAAAGTGACA; 2R, gatcTGTCACTTTATGACAG; 3F, gatcCTGATCTTTTCAATTAGCCTTCCA; 3R, gatcTGGAAGGCTAATTGAAAAGATCAG; 4F, GATCCGGGGCGACTTCCGCCTATTTCCAGAAATTAAGCTCAAACTTGACGTGCAGCTA; 4R, gatCTAGCTGCACGTCAAGTTTGAGCTTAATTTCTGGAAATAGGCGGAAGTCGCCCCG; 5R, CTAGCTGCACGTCAAGTTTGAGC; 3X1F, CTATTTGTCGTGACGATGCAATTTTCCTATTTGTCGTGACGATGCAATTTTCCTATTTGTCGTGACGATGCAATTTTCGAGCT; 3X1R, CGAAAATTGCATCGTCACGACAAATAGGAAAATTGCATCGTCACGACAAATAGGAAAATTGCATCGTCACGACAAATAG; and Sp1 (containing a consensus site for the transcription factor Sp1, shown in boldface type), GATCGATCG**GGGCGGG**GCGATC. The underlined sequences are 5′ overhanging ends compatible with *Bam*HI or *Bgl*II restriction sites.

Construction of CAT reporter plasmids. Plasmids pHP and pHE were constructed by cloning the 9.5-kbp *Hin*dIII-partial *Pst*I and 9-kbp *Hin*dIII-partial *Eco*RV *Pax-QNR* genomic fragments, respectively, into the *Hin*dIII-*Pst*I and *Hin*dIII-blunt-ended *Xba*I sites of the promoterless vectors pBLCAT6 (described in reference 5) and pBLCAT5 (described in reference 37). Construct pP0CAT was obtained by cloning the 1.5-kbp *Hin*dIII-*Bam*HI *Pax-QNR* promoter fragment, excised from the pXA promoter construct (described in reference 31), into the *Hin*dIII-*Bam*HI sites of pBLCAT6. pTKCAT corresponds to the pBLCAT5 vector described in reference 5, which contains the chloramphenicol acetyltransferase (CAT) gene under the control of the TK promoter. pP0CAT EP and pTKCAT EP were constructed by cloning the 460-bp *Eco*RV-blunt-ended *Pst*I fragment, in its normal genomic orientation, downstream of the CAT gene, into the *Sma*I sites of pP0CAT and pTKCAT, respectively. Constructs pTKCAT ED and pTKCAT DP were obtained by blunt-end cloning of the 210-bp *Eco*RV-*Dra*I fragment (corresponding to the 5′ part of the 460-bp *Eco*RV-*Pst*I fragment) and the 250-bp *Dra*I-*Pst*I fragment (corresponding to the 3′ part of the 460-bp *Eco*RV-*Pst*I fragment) 3′ to the CAT gene, respectively, into the *Sma*I site of pTKCAT. Construct pTKCAT P, which contains the mouse *Pax-6* enhancer element, was obtained by cloning the blunt-ended 1-kbp *Pst*I mouse genomic fragment into the *Sma*I site of pTKCAT. DF deletion constructs of the 210-bp *Eco*RV-*Dra*I fragment were created by PCR amplification with the oligonucleotide primers listed above. The individual amplified fragments were inserted into the *Sma*I site of pTKCAT. The resulting plasmids are the following: pTKCAT 1234, a 163-bp fragment containing sites DF1 to DF4 (42 to 204 in Fig. 5; oligonucleotides 1F and 5R); pTKCAT 234, a 125-bp fragment containing sites DF2 to DF4 (80 to 204 in Fig. 5; oligonucleotides 2F and 5R); pTKCAT 34, an 88-bp fragment containing sites DF3 and DF4 (117 to 204 in Fig. 5; oligonucleotides 3F and 5R); pTKCAT 123, a 99-bp fragment containing sites DF1 to DF3 (42 to 140 in Fig. 5; oligonucleotides 1F and 3R); and pTKCAT 12, a 54-bp fragment containing sites DF1 and DF2 (42 to 95 in Fig. 5; oligonucleotides 1F and 2R). To obtain the constructs containing the individual DF cloned in one or multiple copies, the complementary oligonucleotides listed above were annealed, concatemerized, blunt ended, and inserted 3′ to the CAT gene into the blunt-ended *Kpn*I site of the pTKCAT vector. The resulting plasmids are the following: pTKCAT 1, one copy of the 27-bp DF1 fragment (42 to 68 in Fig. 5; oligonucleotides 1F and 1R); pTKCAT (DF2)4, four copies of the 16-bp DF2 fragment (80 to 95 in Fig. 5; oligonucleotides 2F and 2R); pTKCAT (DF3)4, four copies of the 24-bp DF3 fragment (117 to 140 in Fig. 5; oligonucleotides 3F and 3R); pTKCAT 4, pTKCAT (DF4)2, and pTKCAT (DF4)3, one, two, or three copies of the 58-bp DF4 fragment (146 to 204 in Fig. 5; oligonucleotides 4F and 4R). pTKCAT (DF1)3 contains three copies of the 26-bp DF1 fragment (42 to 67) and was constructed by annealing oligonucleotides 3X1F and 3X1R and cloning them into the *Sma*I-*Sac*I sites of pTKCAT. All constructs involving PCR and the cloning of synthetic oligonucleotides were verified by double-stranded dideoxynucleotide sequencing.

Cell culture and transfection. QNR, prepared from 5- or 7-day-old embryos, were seeded at one neuroretina per 35-mm dish in Dulbecco modified Eagle medium–10% fetal calf serum, 24 h prior to transfection. Retinal pigment epithelial cells (RPE), prepared from 7-day-old quail embryos, and QEC were seeded at 1.5×10^5 cells per 35-mm-diameter dish in the same conditions. Transfections were performed by the calcium phosphate method. Cells were transfected with 10 μg of CAT constructs. Each experiment was performed in duplicate with two different DNA preparations, without or with 0.5 μg of plasmid SV-Luc, as an internal control of transfection efficiency. As transfection efficiencies were very similar for all constructs, the results obtained with or without the internal control were identical.

Luciferase and CAT assays. After transfection (48 h), the cells were harvested and lysed in the reporter lysis buffer (Promega). One-tenth of the extract was tested for luciferase activity as described by Crepieux et al. (11). CAT assays were performed on the remaining cell extracts as described by Gorman et al. (19).

RNase protection analysis. Total RNA was extracted from quail embryonic neuroretina at day 5 to day 14 of development by the RNAzol method (9). A 653-bp *Xho*I-*Asp*718 genomic fragment encompassing exon 0 and cloned into the pGEM3 vector (described in reference 31) was used to synthesize the antisense RNA probe, with T7 polymerase in the presence of [α-^{32}P]CTP under conditions provided by the riboprobe system (Promega). RNase protections were performed with 20 μg of RNA as described previously (12), and the protected fragments were separated on a 6% sequencing gel. When the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA probe was used, RNase protections were performed with 2 μg of RNA supplemented to 20 μg with yeast tRNA.

Nuclear run-on experiments. Nuclei were prepared from embryonic day 7 (E7) QNR or RPE and 10-day-old QEC as described by Schibler et al. (34), except that 0.33% (vol/vol) Nonidet P-40 was added to the lysis buffer. The in vitro elongation reaction and labeled RNA extraction were performed as described by Spegelaere et al. (35), except that the nuclei were labeled for 30 min at 30°C. Labeled RNA (10^7 cpm) from E7 QNR, QEC, or RPE nuclei was hybridized at 42°C for 48 h to prehybridized nitrocellulose filters containing 5 μg of test or control denatured plasmid in a solution of 50% formamide, 3× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate), 20 mM $NaPO_4$ (pH 6.5), 1× Denhardt's solution, and 100 μg of *Escherichia coli* tRNA per ml. After hybridization, the filters were washed for 10 min at 50°C in 0.1× SSC, then in 0.3 M NaCl containing 10 μg of RNase A per ml, and again in 0.1× SSC, dried, and autoradiographed. The blots were quantified after exposure of the filters to a PhosphorImager screen. Three plasmids were used: the vector pGEM3; pExon 0, containing the 653-bp *Xho*I-*Asp*718 genomic fragment encompassing exon 0 cloned into the pGEM3 vector; and pGAPDH, containing a 450-bp fragment of the quail GAPDH cDNA that we isolated from a 15-day-old quail spinal cord cDNA library (kindly supplied by C. Dulac).

Nuclear protein preparation. Nuclei were prepared as described above, and nuclear extracts were prepared according to Lavery and Schibler (23) except that these were dialyzed twice for 2 h against DB buffer {20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 60 mM KCl, 0.25 mM EDTA, 0.125 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′,-tetraacetic acid [EGTA], 20% [vol/vol] glycerol, 1 mM dithiothreitol, and a cocktail of proteinase and phosphatase inhibitors described by Carrière et al. [6]} before being stored at −70°C. Final protein concentrations were determined by the Bradford method (Bio-Rad).

Electrophoretic mobility shift assay (EMSA). Double-stranded oligonucleotides were 5′ end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, 5′ overhangs were filled in with the Klenow fragment of DNA polymerase I (Boehringer) and excess deoxynucleotide triphosphates, and the oligonucleotides were purified by electrophoresis on a 20% polyacrylamide gel. Labeled oligonucleotide (1 ng) was incubated on ice for 1 h with 1 μg of nuclear extracts in the presence of 5 μg of poly(dI-dC) in a final volume of 16 μl of 0.5× DB buffer. For competition experiments, a 10- or 100-fold molar excess of unlabeled double-stranded oligonucleotides was added simultaneously with the probes. The DNA-protein complexes were analyzed by electrophoresis on a 6% polyacrylamide gel in 0.25× Tris-borate-EDTA at room temperature for 2 h at 180 V, and the gel was dried for autoradiography.

Southwestern analysis. Southwestern (DNA-protein) blotting was performed with 100 μg of E7 QNR, QEC, or RPE crude nuclear extracts electrophoresed through a sodium dodecyl sulfate (SDS)-polyacrylamide gel. Proteins were electrophoretically transferred to an Immobilon membrane (Millipore) for 5 h at room temperature in the transfer buffer (0.025 M Tris-HCl, 0.192 M glycine, 10% [vol/vol] methanol). After transfer, the membrane was treated as described by Leprince et al. (24), except that the double-stranded probes were obtained as described above and were used at 10^5 dpm per ml.

DNase I footprinting. The footprinting probes were prepared by end labeling of DNA restriction fragments with the Klenow fragment of DNA polymerase I (Boehringer) in the presence of [α-^{32}P]dCTP and [α-^{32}P]dATP (50 μCi each) and purified, after secondary restriction, by electrophoresis on a 5% polyacrylamide gel. End-labeled DNA fragment (5 ng) was incubated on ice for 1 h with 30 or 100 μg of nuclear proteins in the presence of 5 μg of poly(dI-dC) in 50 μl of 0.5× DB buffer supplemented with 5 mM $MgCl_2$ and 2.5 mM $CaCl_2$. After incubation, digestion by 20 ng of DNase I (Bethesda Research Laboratories) was performed for 30 s at 20°C. The reaction was stopped by addition of 30 μl of 50 mM EDTA and 0.5 mg of proteinase K per ml, phenol extracted, and ethanol precipitated. The recovered DNA was then loaded on an 8% sequencing gel.

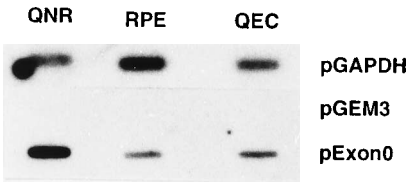

FIG. 1. Nuclear run-on experiments. Shown is an autoradiograph of the hybridization of in vitro transcripts from embryonic E7 QNR (left panel), RPE (middle panel), and QEC (right panel) nuclei. pExon0 contains the 653-bp *Xho*I-*Asp* 718 *Pax-QNR* gene fragment (from −526 to +127 [31]) subcloned into the pGEM3 vector. pGAPDH, containing part of the quail GAPDH cDNA, was included as a positive control, while pGEM3 was used as control for nonspecific hybridization signal.

Nucleotide sequence accession number. The nucleotide sequence data of the quail enhancer are included in the sequence appearing in the EMBL nucleotide sequence database under accession number X68168. The nucleotide sequence of the *Mus musculus* enhancer appears in the EMBL nucleotide sequence database under accession number X82150.

RESULTS

Nuclear run-on experiments. In order to determine whether neuroretina-specific expression of *Pax-QNR* P0-initiated mRNAs is due to transcriptional control, in vitro nuclear run-on assays were performed. We compared embryonic day 7 QNR with QEC and embryonic RPE; cells of the latter two types do not express these mRNAs. Thus, nuclei isolated from these cells were incubated with nucleoside triphosphates in the presence of [^{32}P]UTP, and the nuclear RNA was extracted. An equivalent amount of labeled nuclear RNA from each cell type was hybridized to filters containing the first exon of the *Pax-QNR* gene (exon 0, [13]) cloned in the plasmid vector pGEM3 (pExon0). The controls used included plasmid vector pGEM3 and a plasmid containing a fragment of the quail GAPDH cDNA. The results (Fig. 1) show a higher level of transcripts of the *Pax-QNR* gene in E7 QNR nuclei than in QEC or RPE nuclei, whereas transcription of the GAPDH gene was similar in all three cells. No significant hybridization to a probe upstream of the transcription initiation site (data not shown) was found. Therefore, these data demonstrate that transcriptional regulation takes place in the neuroretina-specific expression of MC29-QNR2 mRNA.

Identification of a neuroretina-specific 3′ enhancer within the *Pax-QNR* gene. Previous studies have shown that the −1386 to +127 promoter sequences function relatively equally in both QNR and QEC (31). Thus, additional upstream and/or downstream regulatory elements might be responsible for upregulating the transcription of the *Pax-QNR* gene in neuroretina cells. To locate potential *cis* control regions regulating the neuroretina-specific transcription of the P0-initiated mRNAs, we tested 5′- and 3′-flanking promoter DNAs for their ability to enhance transcription from the promoter in neuroretina cells. Among the 3′-flanking promoter DNA CAT constructs tested (data not shown), two retained our attention: the pHP and pHE constructs (shown in Fig. 2A), which contain about 8 and 7.5 kbp, respectively, of 3′-flanking promoter sequences upstream of the CAT gene. After transient transfection of these constructs into E7 QNR cells, pHP showed CAT activity (about 8-fold) higher than that observed for the deletion construct pHE (Fig. 2A). This suggests that the 460-bp *Eco*RV-*Pst*I fragment, located 7.5 to 8 kbp 3′ to the *Pax-QNR* P0 promoter and deleted in the pHE construct, contains DNA sequences that may increase expression of the reporter gene.

To determine whether this region contains a neuroretina-specific transcriptional enhancer element, the 460-bp *Eco*RV-*Pst*I fragment was subcloned, in its normal genomic orientation, 3′ to the CAT gene in the pP0CAT and pTKCAT vectors, in which the CAT reporter gene is driven by the *Pax-QNR* P0 promoter (31) or the minimal TK promoter (vector pBLCAT5 [5]), respectively (Fig. 2B). The resulting constructs (pP0CAT EP and pTKCAT EP; Fig. 2B) and their parental plasmids pP0CAT and pTKCAT were transiently transfected into E7 QNR, QEC, and RPE and assayed for CAT activity. In this case, the CAT activities of the chimeric CAT constructs were expressed relative to the values for the parental plasmids. The results show (Fig. 2B) that in E7 QNR, the 460-bp *Eco*RV-*Pst*I fragment enhanced CAT activity by the *Pax-QNR* P0 and TK promoters by about 6-fold above the levels of the vectors without enhancer. However, in QEC and RPE, which do not express the P0-initiated mRNAs, the *Eco*RV-*Pst*I fragment failed to enhance CAT activity by the *Pax-QNR* P0 or TK promoters above basal levels (Fig. 2B). The enhancer activity of the 460-bp *Eco*RV-*Pst*I fragment was observed to be slightly lower (about twofold) when assayed in the opposite orientation (data not shown). No enhancer activity was detectable when this fragment was inserted 3′ to the CAT gene in the promoterless pBLCAT6 vector (data not shown). These results demonstrate that the 460-bp *Eco*RV-*Pst*I fragment contains a functional neuroretina-specific enhancer element. The activity of this 3′ enhancer is not restricted to the *Pax-QNR* P0 promoter and is orientation independent.

In order to characterize more precisely the enhancer element, deletions from the 5′ and 3′ ends of the *Eco*RV-*Pst*I enhancer fragment were generated and tested for enhancer activity after transient transfection into E7 QNR. The results show (Fig. 2C) that the 5′ portion of the *Eco*RV-*Pst*I fragment (construct pTKCAT ED containing the 210-bp *Eco*RV-*Dra*I fragment) retained the full enhancer activity of the *Eco*RV-*Pst*I fragment (compare constructs pTKCAT ED and pTKCAT EP). In contrast, the 3′ portion of the *Eco*RV-*Pst*I fragment (construct pTKCAT DP containing the 250-bp *Dra*I-*Pst*I fragment) had no enhancer activity (compare pTKCAT DP and pTKCAT). Thus, the enhancer element is located more precisely in a 210-bp *Eco*RV-*Dra*I fragment.

The neuroretina-specific 3′ enhancer functions in a developmental stage-dependent manner. We have previously shown that the level of *Pax-QNR* mRNAs, monitored by Northern (RNA) blot analysis, varied during the development of the embryonic avian neuroretina, increasing from day 5 to day 8, when it reaches a plateau (27). Since the *Pax-QNR* gene expresses multiple mRNAs differing by their 5′ untranslated region (13), we examined the pattern of expression of the P0-initiated mRNAs during the embryonic quail neuroretina development by RNase protection assays. An antisense labeled RNA probe encompassing exon 0 (described in reference 31 and used to determine the P0 transcriptional start site) was hybridized to total RNAs extracted from embryonic quail neuroretina at day 5 (E5) until day 14 (E14) of development. Results show (Fig. 3A) that the level of P0-initiated mRNAs (indicated by a bracket) was very low in E5 neuroretina, increased markedly from E5 until E8, and remained constant until E14. As a control, the levels of GAPDH mRNA were monitored and were similar among these samples (Fig. 3B). These data are reminiscent of those obtained previously by Northern blot analysis (27).

The low level of P0-initiated mRNAs detected in E5 neuroretina, followed by a marked increase until E8, prompted us to investigate whether the enhancer activity varies with development. Thus, the enhancer constructs described in Fig. 2B and their parental plasmids were transiently transfected into E5 and E7 QNR and assayed for CAT activity. The CAT activities

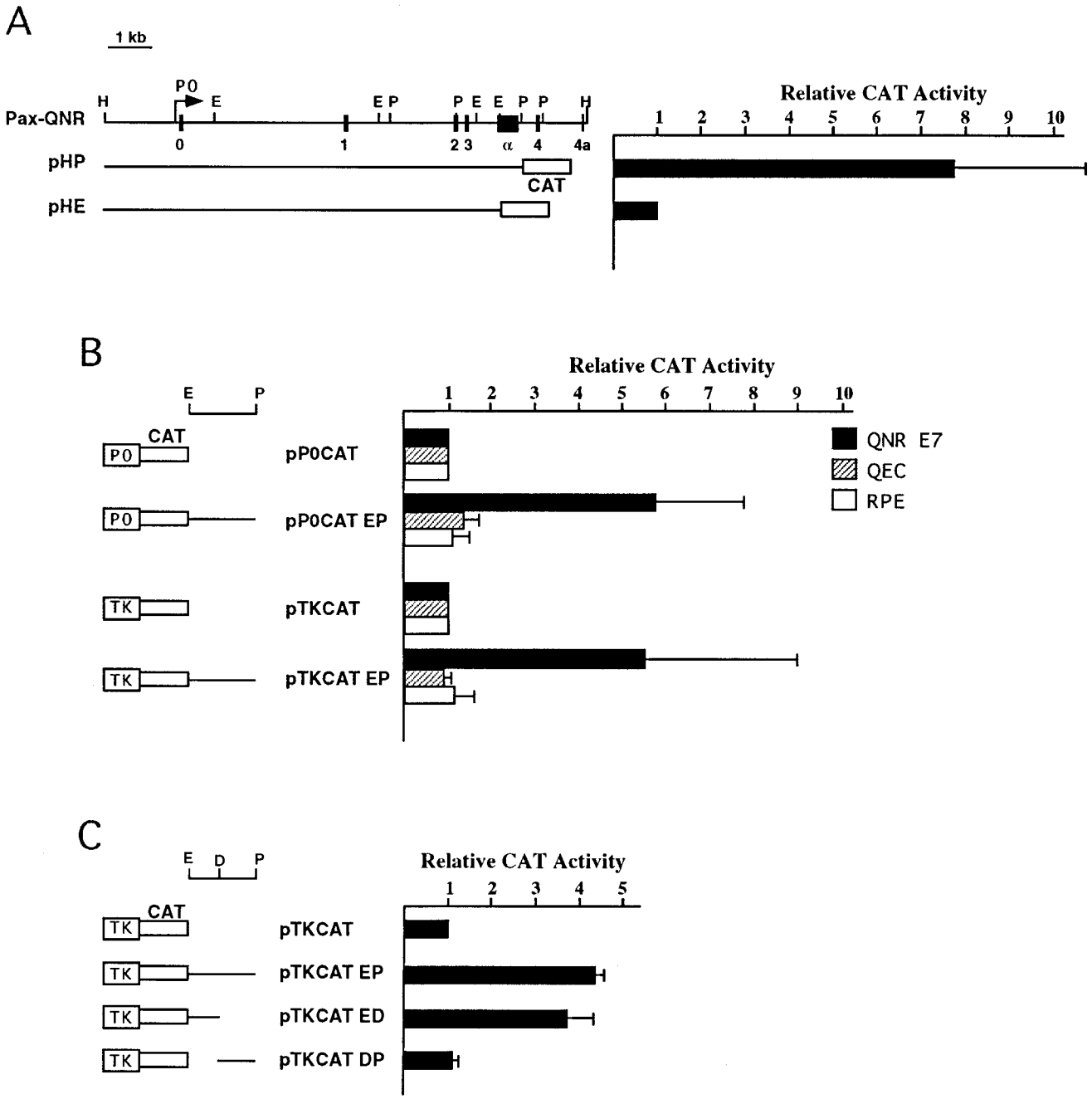

FIG. 2. Identification of a neuroretina-specific 3′ enhancer element within the *Pax-QNR* gene. (A) Test of *Pax-QNR* 3′ regions for the presence of *cis* regulatory regions. The top line shows a partial map of the *Pax-QNR* gene with the exons boxed. The positions of the *Hin*dIII (H), *Pst*I (P), and *Eco*RV (E) restriction enzyme sites used to generate the CAT constructs are marked. pHP and pHE CAT constructs contain the 9.5-kbp partial *Hin*dIII-*Pst*I and the 9-kbp partial *Hin*dIII-*Eco*RV fragments, respectively, cloned upstream of the CAT gene. These constructs were transiently transfected into E7 QNR cells, and levels of CAT activity were quantitated after exposure of the thin-layer chromatograms to a PhosphorImager screen. CAT activity is expressed relative to that of the pHE construct, chosen as a reference and arbitrarily set at 1. (B) The *Pax-QNR* 3′ region contains a neuroretina-specific enhancer functional on the *Pax-QNR* P0 or TK promoter. pP0CAT EP and pTKCAT EP contain the 460-bp *Eco*RV-*Pst*I fragment inserted in the natural orientation, 3′ to the CAT gene in plasmids pP0CAT and pTKCAT, respectively. These plasmids were transiently transfected into E7 QNR, QEC, and RPE, and CAT activities were measured with an Instant Imager (Packard). CAT activities of the enhancer constructs are expressed relative to those of the parental plasmids. (C) Delineation of the minimum enhancer element. The position of the *Dra*I (D) restriction enzyme site used to generate the deletion constructs is marked. pTKCAT ED and pTKCAT DP contain the 210-bp *Eco*RV-*Dra*I (5′ portion of the *Eco*RV-*Pst*I sequence) and the 250-bp *Dra*I-*Pst*I (5′ portion of the *Eco*RV-*Pst*I sequence) fragments, respectively, cloned in the natural orientation 3′ to the CAT gene in plasmid pTKCAT. After transient transfection of these constructs into E7 QNR cells, CAT activities were quantified as for panel A. CAT activities of the constructs are expressed relative to that of the pTKCAT plasmid. The results shown in all three panels are the averages of at least two independent transfection experiments performed in duplicate with two different DNA preparations, with the standard deviations indicated by error bars.

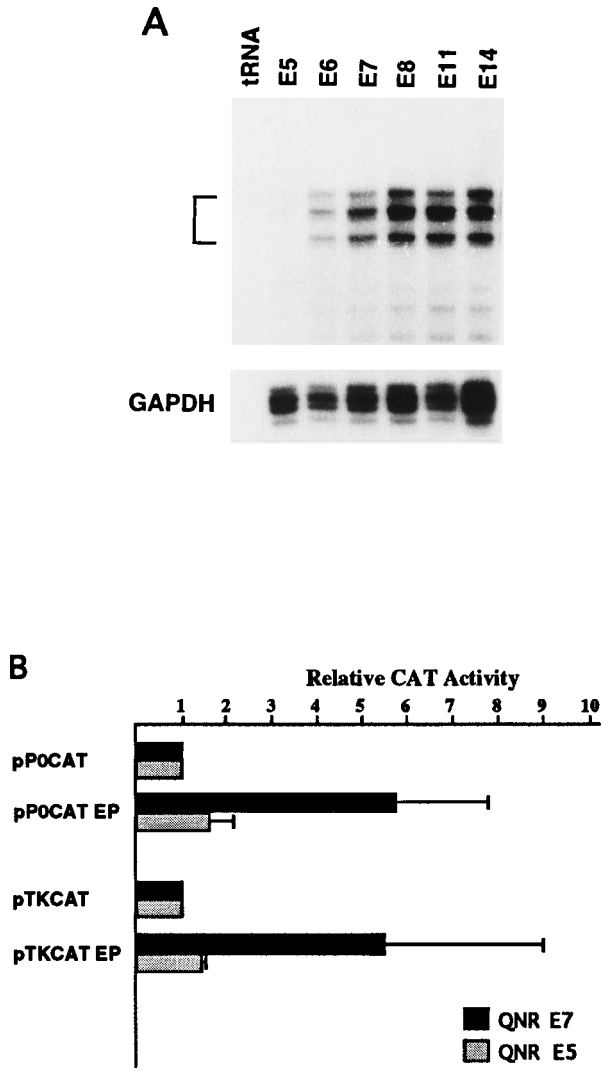

FIG. 3. Developmental analysis of the P0-initiated mRNAs and of the 3′ enhancer activity. (A) RNase protection analysis. Total RNA (20 μg) from QNR at stages E5 to E14 was hybridized with an antisense RNA probe encompassing exon 0 (described in reference 31). Following RNase digestion, the samples were analyzed on a sequencing gel. The *Pax-QNR* mRNAs initiated at the P0 promoter and protected by the probe are indicated by a bracket. The probe (top of the gel) is not shown. tRNA was included as a negative control. As a control, hybridization of the mRNAs with an antisense GAPDH RNA probe is shown (bottom panel). (B) Functional analysis of the 3′ enhancer in neuroretina at different developmental stages. The CAT constructs were transiently transfected into E5 and E7 QNR, and CAT activities were measured with an Instant Imager (Packard). CAT activities of the enhancer constructs are expressed relative to those of the parental plasmids. The results shown are the averages of three independent transfection experiments performed in duplicate with two different DNA preparations, with the standard deviations indicated by error bars.

of the enhancer CAT constructs were expressed relative to the values for the parental plasmids. The results show (Fig. 3B) that, in contrast to E7 QNR in E5 QNR the enhancer element increased CAT activity by the *Pax-QNR* P0 or TK promoters very weakly above basal levels. These data indicate that the activity of the 3′ enhancer in embryonic neuroretina is developmentally controlled and suggest that its increase between E5 and E7 could be responsible for the variation in the level of P0-initiated mRNAs observed between these two stages.

Footprinting studies. The neuroretina-specific 3′ enhancer was located to a 210-bp *Eco*RV-*Dra*I fragment (Fig. 2C). In DNase I footprinting experiments, we investigated whether this sequence could be specifically recognized in vitro by *trans*-acting factors. The *Eco*RV-*Pst*I fragment, end labeled on the noncoding strand, was incubated with either E7 QNR, QEC, or RPE nuclear extracts and subjected to moderate DNase I digestion. With E7 QNR nuclear extracts, four protected regions, named DNase I footprints (DF) DF1, DF2, DF3, and DF4, were observed in the *Eco*RV-*Dra*I fragment (Fig. 4). The footprints were faint when 30 μg of nuclear extracts was used (lanes 1) but were much more visible when 100 μg of nuclear extracts was used (lanes 2). The 5′ border of DF1 is not clearly visible in Fig. 4; however, this footprint was much more delineated in other experiments (data not shown). Footprint locations and sequences are shown in Fig. 5. With QEC and RPE nuclear extracts, the four footprints observed with E7 QNR nuclear extracts were not found (Fig. 4B). It is noteworthy that with QEC nuclear extracts a protected region extending 5′ of and partially overlapping DF4 was observed (Fig. 4B), suggesting that a different factor binds this region. A footprinting experiment performed with a probe overlapping the *Eco*RV site failed to reveal any further protected region between DF1 and this site (data not shown). These results demonstrate the presence of four neuroretina-specific nuclear protein-binding sites in the 3′ enhancer element.

Deletional analysis of the neuroretina-specific enhancer. To test directly the role of these protein-binding sequences in the neuroretina-specific enhancer activity, we constructed DF deletion mutants of the 210-bp *Eco*RV-*Dra*I fragment by PCR amplification (summarized in Fig. 6A). The constructs were transiently transfected into E7 QNR cells and assayed for enhancer function. In this case, the enhancer activities of the constructs were expressed relative to the value for the full-size enhancer construct pTKCAT ED (set at a value of 100). Results show (Fig. 6A) that a 163-bp fragment extending through the four footprints, construct pTKCAT 1234, retained the full enhancer activity. Deletion of site DF1 (construct pTKCAT 234) or DF4 (construct pTKCAT 123) strongly reduced the enhancer activity. However, a single copy of either DF1 (construct pTKCAT 1) or DF4 (pTKCAT 4) mediated a very weak enhancer activity, if any. Additional deletion of DF2 (pTKCAT 34 and pTKCAT 1) or DF3 (pTKCAT 12 and pTKCAT 4) also decreased the enhancer activity, suggesting that these binding sites contribute to the enhancer activity. We cloned multiple copies of the individual DF sites, 3′ to the CAT gene in the pTKCAT vector (summarized in Fig. 6B). After transient transfection in E7 QNR, the CAT activities of the constructs were expressed relative to the value for the pTKCAT vector. Results show (Fig. 6B) that when multiple copies of either the DF1, DF2, or DF3 site were fused with the TKCAT [pTKCAT (DF1)3, pTKCAT (DF2)4, and pTKCAT (DF3)4], a weak enhancement, if any, was observed. In contrast, whereas a single copy of the DF4 site (pTKCAT 1) had no enhancer activity, multiple copies of the DF4 site [pTKCAT (DF4)2 and pTKCAT (DF4)3] mediated enhancer activity, which increased with the number of the copies cloned. Thus, multimerization of the DF4 site mimics the enhancer activity, suggesting that, in this context, DF4 binds *trans*-acting factors able to mediate the enhancer activity. Since, in the natural enhancer element, maximal enhancing occurs when the four binding sites are present, we conclude that the DF sites cooperate to mediate full enhancer activity.

Characterization of nuclear protein binding to individual DF within the neuroretina enhancer. To further characterize individual protein-binding sites within the neuroretina-specific enhancer, EMSA were performed with double-stranded labeled oligonucleotides corresponding to DF1, DF2, DF3, and

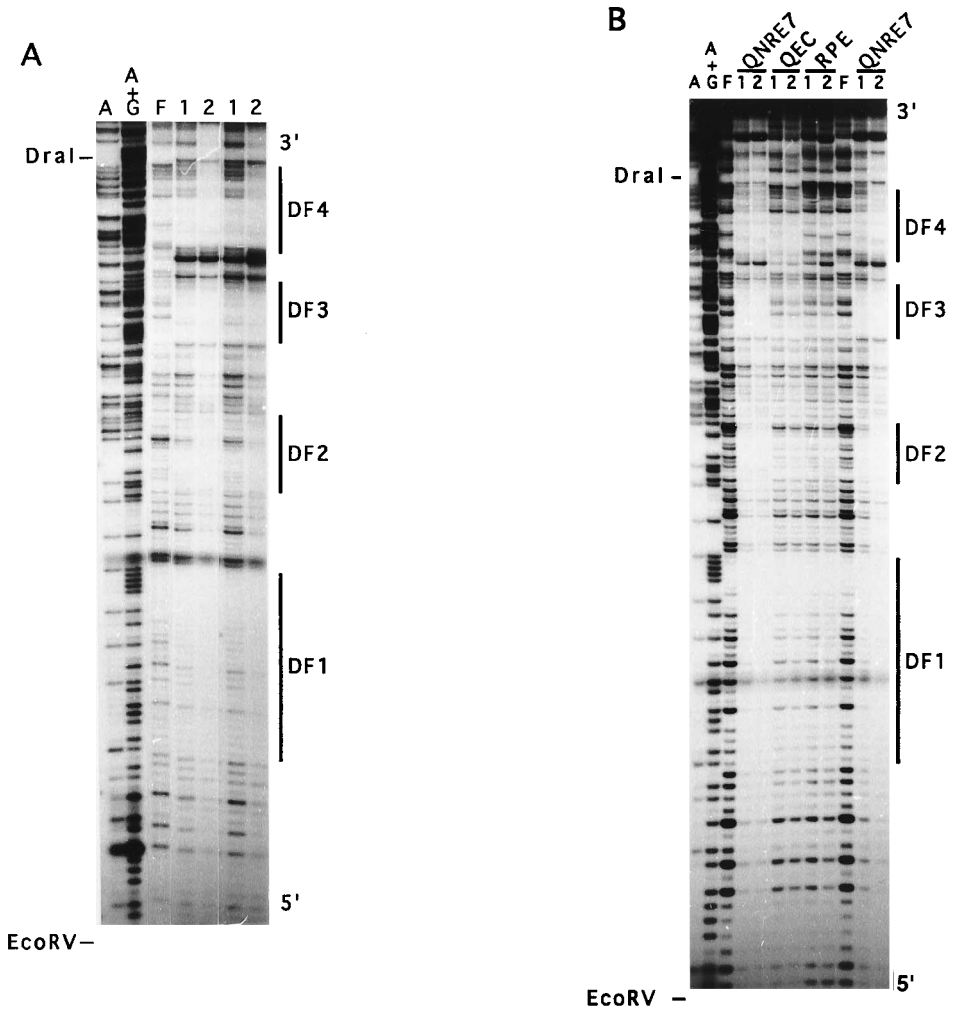

FIG. 4. Footprinting studies. (A) DNase I footprinting analysis of the *Eco*RV-*Pst*I fragment in the absence (F) or presence of 30 μg (lanes 1) or 100 μg (lanes 2) of E7 QNR nuclear extracts. (B) DNase I footprinting analysis of the *Eco*RV-*Pst*I fragment in the absence (F) or presence of 30 μg (lanes 1) or 100 μg (lanes 2) of nuclear extracts from E7 QNR, QEC, or RPE. For both panels, the positions of the *Eco*RV and *Dra*I restriction sites, delineating the 5′ and 3′ borders, respectively, of the enhancer element, are indicated. The positions of the DF referred to in the text are also marked. Lanes A and A+G, sequencing reactions on the probe.

DF4 (defined in Fig. 5; also see Materials and Methods) and with nuclear extracts from E7 and E5 QNR, as well as from QEC and RPE (Fig. 7A). When the oligonucleotides were incubated with E7 QNR nuclear extracts, multiple complexes were formed (Fig. 7A, lanes 1, and Fig. 8, lanes 2), some of which were discrete (indicated by small arrows). The competition experiments presented in Fig. 8 show that these complexes were specific, since their formations were inhibited or strongly reduced by the presence of a 100-fold excess of the corresponding unlabeled oligonucleotide (for probe DF1, see Fig. 8A, lane 4; for probe DF2, see Fig. 8B, lane 6; for probe DF3, see Fig. 8C, lane 8; and for probe DF4, see Fig. 8D, lane 10), but not by the presence of a similar excess of a nonspecific competitor corresponding to the Sp1 consensus binding site (Fig. 8, lanes 12). These complexes were also detected, albeit at lower levels, with E5 QNR nuclear extracts (Fig. 7A, lanes 4). This might suggest that the factors binding to the oligonucleotides are less abundant in neuroretina at day E5 of development or that the affinity of these factors to their sequences is lower. These results might explain the differential enhancer activity observed between E5 QNR and E7 QNR (Fig. 3B). These complexes were not detected with the QEC or RPE

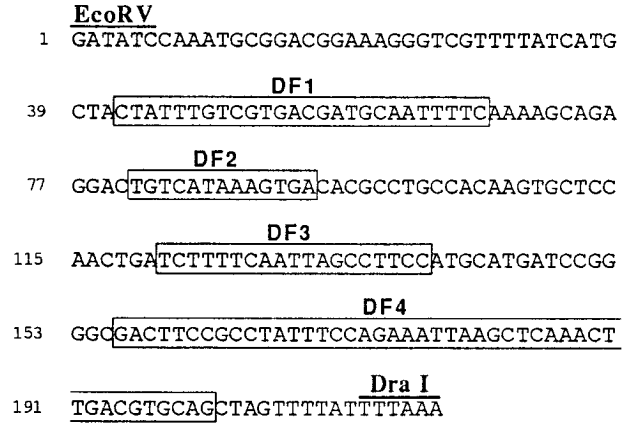

FIG. 5. Nucleotide sequence of the 3′ enhancer element. The sequence of the 216-bp *Eco*RV-*Dra*I fragment is given, with the four neuroretina-specific protein-binding sites (DF) boxed. Restriction sites used for subcloning are indicated. Nucleotide numbers are shown on the left.

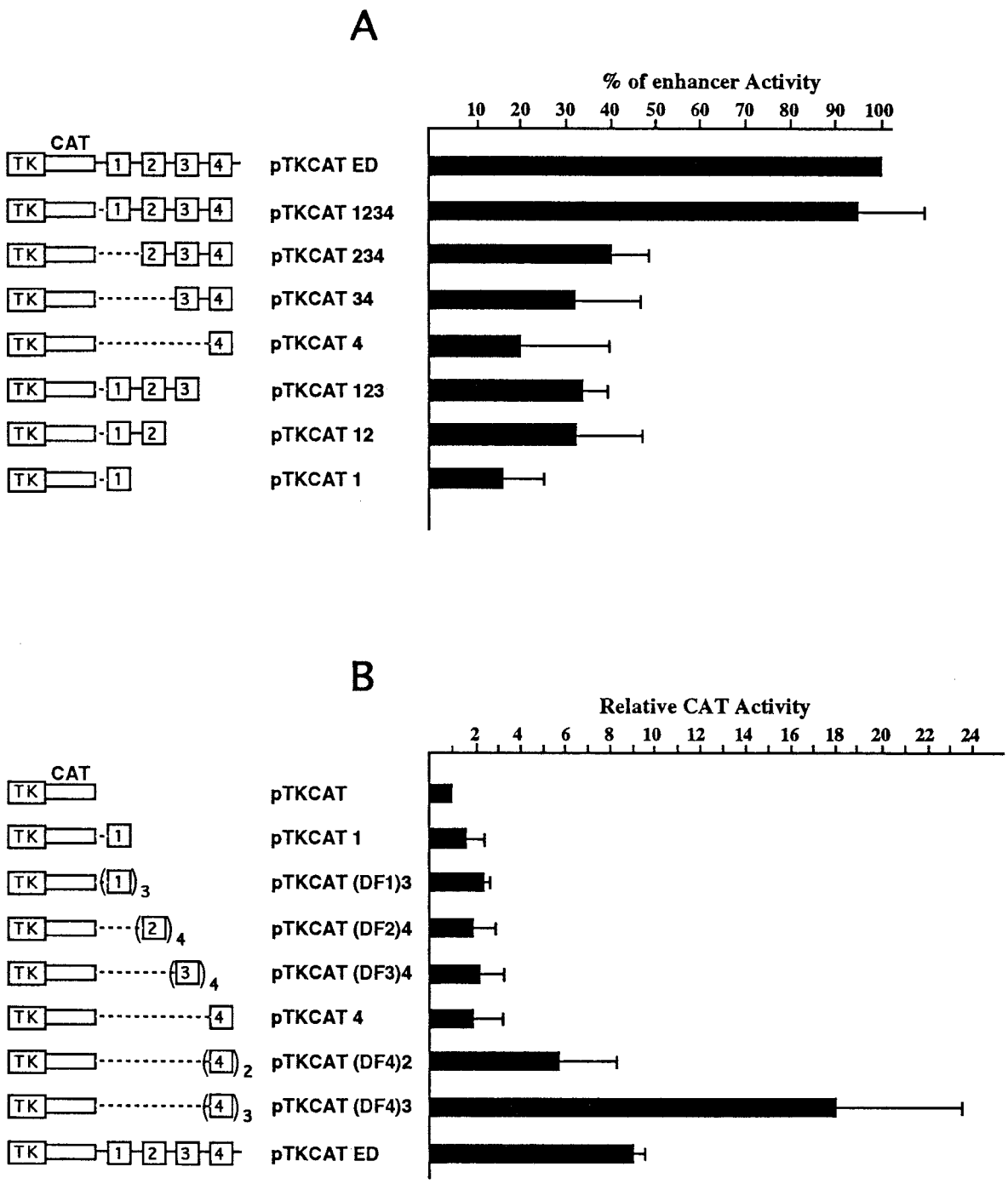

FIG. 6. Role of the four neuroretina-specific protein-binding sequences in the enhancer activity. (A) Deletional analysis of the neuroretina-specific enhancer. The DF deletion constructs, listed in the center of the panel, were transiently transfected into E7 QNR cells, and CAT activities were measured as for Fig. 2A. The CAT activities of the DF deletion constructs were expressed relative to that of the full-size enhancer construct pTKCAT ED (set at a value of 100). (B) Biological activity of each protein-binding site. Multiple copies of either DF1, DF2, DF3, or DF4 were cloned, 3′ to the CAT gene, in the pTKCAT vector. The resulting constructs, listed in the center of the panel, were transiently transfected into E7 QNR cells, and CAT activities were measured as for Fig. 2A. CAT activities of the constructs are expressed relative to that of the parental plasmid pTKCAT. The results shown in both panels are the averages of at least three independent transfection experiments performed in triplicate with three different DNA preparations, with the standard deviations indicated by error bars.

nuclear extracts (except with the DF2 probe, in which faint complexes were observed with RPE nuclear extracts; see Fig. 7A). Incubation of QEC or RPE nuclear extracts with oligonucleotides containing consensus binding sites for Ap1 or upstream stimulatory factor indicated that these nuclear extracts were fully functional (data not shown). These results are consistent with the footprinting experiments and confirm the binding of neuroretina-specific factors to the enhancer element.

To identify the nuclear proteins that interact with the neuroretina-specific enhancer, Southwestern analyses were per-

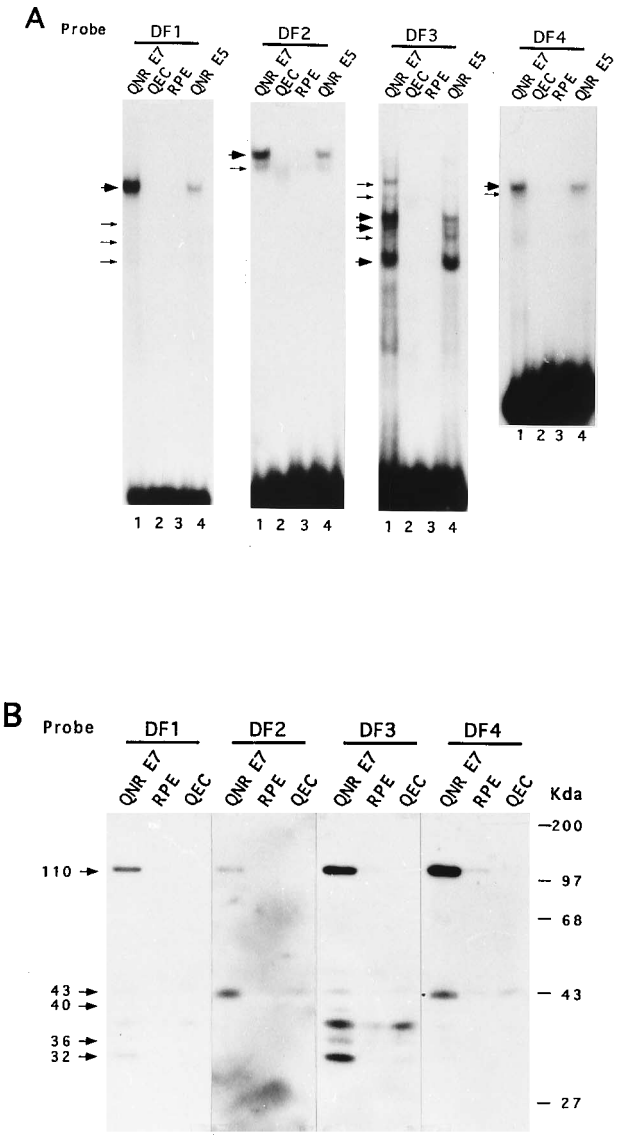

FIG. 7. Characterization of nuclear protein binding to individual DF within the neuroretina enhancer. (A) EMSA analysis of the four protein-binding sites. EMSAs were performed with the radiolabeled DF1, DF2, DF3, and DF4 oligonucleotides, and 1 μg of nuclear extracts from E7 QNR (lanes 1), QEC (lanes 2), RPE (lanes 3), or E5 QNR (lanes 4). The arrows indicate bands of altered mobility representing specific protein-DNA complexes. Big arrows, major complexes; small arrows, minor complexes. (B) Identification of nuclear proteins interacting with the four DF by Southwestern analysis. Nuclear extracts (100 μg) from E7 QNR, QEC, or RPE were subjected to SDS-PAGE. After protein transfer, the labeled DF1, DF2, DF3, and DF4 oligonucleotides were incubated with the renatured nuclear proteins. The potential proteins interacting with the probes are indicated by arrows.

formed. E7 QNR, QEC, or RPE nuclear proteins were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE), immobilized onto nylon membrane, and probed with equal radiospecific activity probes of DF1, DF2, DF3, and DF4. Results show (Fig. 7B) that the four probes bound multiple neuroretina nuclear proteins, albeit with different affinities (if we compare the differences in the intensities of the multiple proteins detected with one probe considered). These results are consistent with the EMSA data showing multiple complexes formed when these probes were incubated with QNR nuclear extracts (Fig. 7A). Two proteins, of 110 and 43 kDa, were bound by all four nucleotides, whereas DF1 and DF3 bound additional proteins of equal molecular masses. These proteins were essentially detected in neuroretina and very faintly in RPE (see Discussion for explanation) and could correspond to the proteins bound to the enhancer element. These results suggest that the four nuclear protein-binding sites detected in the neuroretina-specific enhancer bind a set of proteins, some of which are identical.

To investigate whether identical proteins are present in the complexes detected by EMSAs with probe DF1, DF2, DF3, or DF4 and E7 QNR nuclear extracts (Fig. 7A), we performed competition studies. Competition results are presented in Fig. 8. The major complex formed with the DF1 probe (Fig. 8A, lane 2) was unexpectedly increased by a 100-fold molar excess of either DF2 (lane 6) or DF3 (lane 8), whereas it was partially inhibited by a similar excess of DF4 (lane 10); however, the minor complexes formed with this probe were inhibited by an excess of either DF2, DF3, or DF4. The two complexes formed with the DF2 probe (Fig. 8B, lane 2) were inhibited totally by a 100-fold molar excess of DF4 (lane 10) but only partially by a similar excess of either DF1 (lane 4) or DF3 (lane 8). Likewise, the complexes obtained with the DF3 probe (Fig. 8C, lane 2) were partially inhibited by a 100-fold molar excess of either DF1 (lane 4), DF2 (lane 6), or DF4 (lane 10). Finally, only the minor complex formed with the DF4 probe (Fig. 8D, lane 2) was inhibited by a 100-fold molar excess of either DF1 (lane 4), DF2 (lane 6), or DF3 (lane 8). The specificity of these competition experiments was demonstrated by the fact that the Sp1 oligonucleotide failed to compete with DF1, DF2, DF3, or DF4 for binding (Fig. 8, lanes 11 and 12). The competition results demonstrate that the four DF oligonucleotides are able to modulate the binding among them. These data are consistent with the Southwestern blot results and indicate that some protein-DNA complexes visualized by EMSAs involved a set of identical neuroretina factors. However, these common factors are expected to bind to the four DF with different affinities, as suggested by the partial or total competition obtained.

Our gel retardation assays revealed a complex pattern of protein-DNA interactions involving different as well as common factors, prompting us to compare the sequences of the four neuroretina protein-binding sites. Results show (Fig. 9) that these AT-rich oligonucleotides exhibited some stretches of homologies, but these do not correspond to already described protein-binding sites. However, as pointed out by a referee, octamer-like and CREB-like proteins (binding sites ATGCAAAT and TGACGTA/CA/G, respectively) may be involved.

Nucleotide sequence and enhancer activity of the 3′-flanking homologous region of the mouse *Pax-6* gene. To help determine whether the 3′ quail enhancer is phylogenetically conserved, we isolated a mouse *Pax-6* clone from a genomic library using the quail *Eco*RV-*Pst*I fragment as a probe. The sequence of the mouse homologous region was determined and compared with the sequence of the quail *Pax-6* enhancer determined above (Fig. 5). The two noncoding regions exhibited very high nucleotide sequence identity (89.8%) (Fig. 10A), with the four protein-binding sites identified previously on the quail *Pax-6* enhancer being extremely well conserved: DF3 and DF4 exhibited 100% nucleotide identity, whereas DF1 and DF2 exhibited 88.5 and 92.3% nucleotide identities, respectively. It is striking that of the 22-bp differences between the quail and the mouse sequences, most of them (16) are outside the four binding sites described in the quail *Pax-6* enhancer, suggesting a functional role for these motifs. These results suggest that there could be an active enhancer within the mouse *Pax-6* gene.

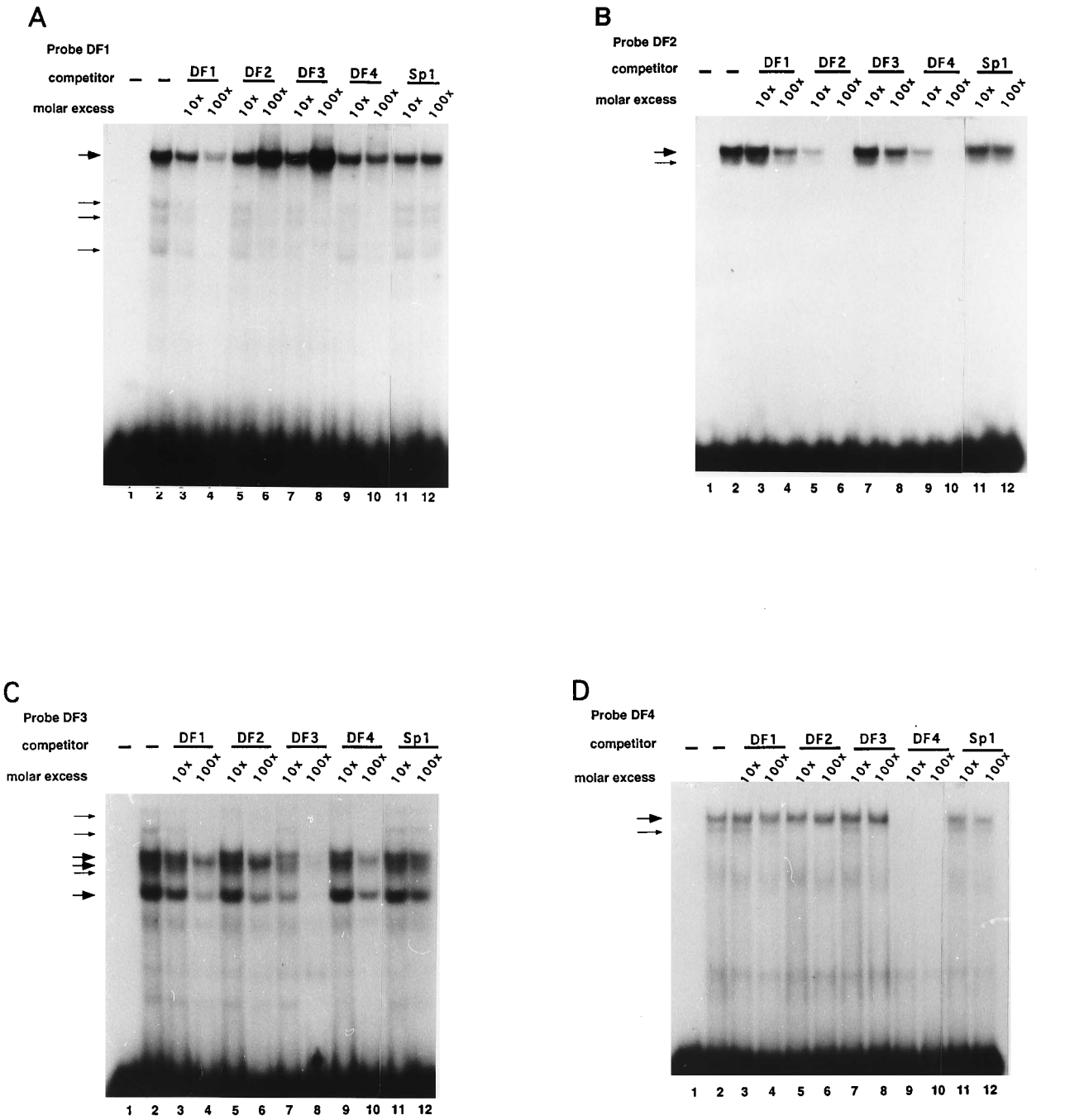

FIG. 8. EMSA analysis of the four neuroretina protein-binding sites. Labeled oligonucleotides DF1 (A), DF2 (B), DF3 (C), and DF4 (D) were incubated either alone (lanes 1) or with 1 μg of E7 QNR nuclear extracts (lanes 2 to 12) in the absence (lanes 2) or in the presence (lanes 3 to 12) of the indicated (fold) molar excess of various unlabeled competitors: DF1 (lanes 3 and 4), DF2 (lanes 5 and 6), DF3 (lanes 7 and 8), DF4 (lanes 9 and 10), and Sp1 (lanes 11 and 12).

To test this hypothesis, a 1-kbp *Pst*I mouse fragment containing the putative enhancer was inserted 3′ to the CAT gene in the pTKCAT vector, yielding pTKCAT P. This construct was transiently transfected into E7 QNR and tested for enhancer activity. As a positive control, we used the quail homologous pTKCAT ED construct. Results show (Fig. 10B) that the 1-kbp *Pst*I mouse fragment (construct pTKCAT P) exhibited an enhancer activity, albeit 2-fold lower than that produced by the quail *Pax-6* enhancer (construct pTKCAT ED). The ability of the mouse fragment to enhance CAT activity from the TK promoter in QNR demonstrates that this region contains a functional enhancer element.

DISCUSSION

In this study, we have demonstrated, by nuclear run-on experiments, that neuroretina-specific expression of *Pax-QNR* P0-initiated mRNAs is, in part, the result of transcriptional

FIG. 9. Nucleotide sequence comparison of the four protein-binding sites DF1, DF2, DF3, and DF4. The stretches of homologies are boxed. Gaps, indicated by dots, were created to maximize the alignment of conserved sequences.

control, and we have identified a neuroretina-specific 3′ enhancer within the *Pax-QNR* gene, 7.5 kbp downstream from the P0 promoter. This neuroretina-specific enhancer exerts an enhancer activity on the homologous P0 *Pax-QNR* or heterologous TK promoters in a position- and orientation-independent manner. Moreover, the activity of the 3′ enhancer in embryonic neuroretina is developmentally controlled and parallels the level of expression of P0-initiated mRNAs. Thus, the increase in the level of P0-initiated mRNAs during the embryonic neuroretina development could be attributed, in part, to the activity of this enhancer.

We have found, both in a DNase I footprinting assay and in an EMSA, that the enhancer element contains four neuroretina-specific nuclear protein-binding sites, and we also demonstrated a functional role for these binding sites in a reporter-based expression assay. The protein-DNA complexes visualized in EMSA are detected at lower levels with E5 QNR nuclear extracts, and this is correlated with a lower enhancer activity at this stage. This might suggest that the factors binding to the enhancer are less abundant in neuroretina at day E5 of development or that the affinity of these factors to their sequences is lower as a result of posttranslational modification, for example. On the basis of dissection of the enhancer region, we propose that the enhancer activity is mediated by a synergy between the four protein-binding sites. Thus, the factors bound to these sites must cooperate with each other in order to activate the neuroretina enhancer. The picture of a tissue-specific enhancer made of several interacting enhansons agrees with the modular nature of the enhancers described so far and reveals similarities with the control elements regulating the tissue-specific expression of other genes (20, 22, 28). Southwestern analysis and EMSA competition experiments demonstrate that the four sites bind a set of unknown identical proteins. Although these proteins are detected essentially in the neuroretina, they are also faintly detected in RPE. One explanation could be the fact that the retinal pigment epithelium possesses the capacity to transdifferentiate into neuroretina in vivo (10) and to generate cells with neuronal traits in certain culture conditions (29, 32; for a review, see reference 15).

However, several questions remain unanswered. Why do the multimerized DF1, DF2, and DF3 sites have weak or no enhancer activity, although they bind factors some of which are identical to those bound by the active multimerized DF4 site? We might suggest different hypotheses. Since EMSAs reveal different protein-DNA complexes between the four DF, perhaps additional proteins (not detectable by Southwestern blotting) are present in the complexes observed with the DF4 fragment and are important to in vivo enhancer activity. That additional proteins are present in the DF4 complexes is strongly suggested by the fact that in EMSA competition experiments only the minor complex is inhibited by unlabeled DF1, DF2, or DF3 fragments. Alternatively, the geometric spacing of the *cis* elements is important for the enhancer activity and this is not respected in the multimerized DF1, DF2, or DF3 fragments. Characterization of the factors involved should provide an answer to these questions. The nucleotide sequences of the four binding sites suggest the possibility that the DNA-binding proteins involved recognize AT-rich boxes and therefore belong to the homeodomain-containing transcription factors (for a review, see reference 14). Such proteins

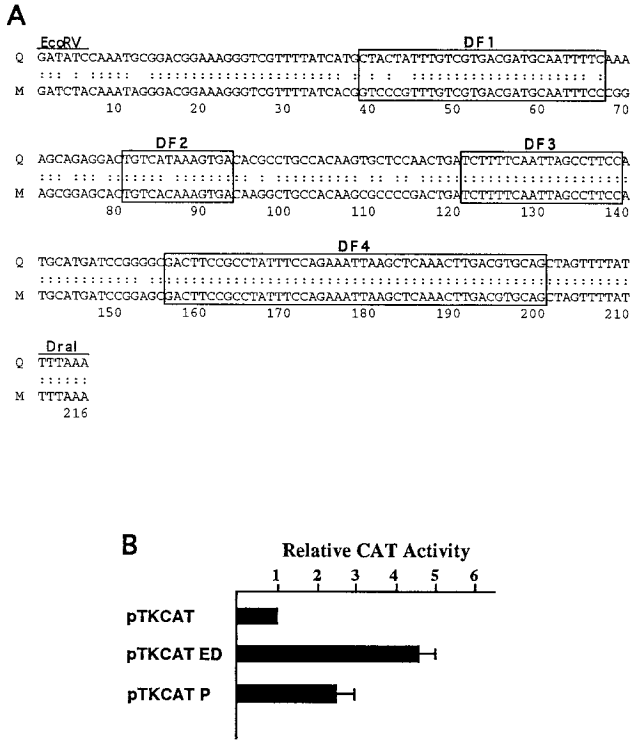

FIG. 10. Nucleotide sequence and enhancer activities of the 3′ flanking homologous region of the mouse *Pax-6* gene. (A) Nucleotide sequence homology between the quail *Pax-6* enhancer (Q) and the homologous region in the mouse *Pax-6* gene (M). Double dots indicate identity of bases at the positions shown. The four protein-binding sequences observed previously on the quail *Pax-6* enhancer are boxed. (B) Enhancer activities of the 3′ flanking homologous region of the mouse *Pax-6* gene. The pTKCAT P and pTKCAT ED constructs were transiently transfected into E7 QNR, and CAT activities were measured as for Fig. 2A. CAT activities of the constructs are expressed relative to that of the parental plasmid pTKCAT. The results shown are the averages of three independent transfection experiments performed in duplicate with two different DNA preparations, with the standard deviations indicated by error bars.

are effectively expressed in the eye (3, 26) and might be involved in *Pax-6* gene regulation.

Nucleotide sequence identity between the quail enhancer and the homologous murine region is approximately 90%, and the four protein-binding sites are also found in the murine sequence. Like the quail sequence, the murine homologous region also exhibits an enhancer activity when tested in transient transfection assays into neuroretina cells. However the mouse enhancer activity is 2-fold lower than that of the quail, despite the great degree of identity of the four protein-binding sites. One explanation could be point mutations in important sequences: the DF1 and DF2 sites differ by 5 and 1 bp from their quail homologs, respectively. Alternatively, there could be an inhibitory sequence, outside the enhancer region, within the 1-kbp mouse genomic fragment cloned in pTKCAT.

Taken together, our results indicate that the neuroretina enhancer described in this study is conserved in mammalian species and is a complex transcriptional regulatory element that potentially contributes to the neuroretina specificity of *Pax-6* gene expression. In this respect, it is interesting to note that the enhancer has no activity in pancreatic cell line βTC-3, which also expresses the *Pax-6* gene (42), and that Southwestern experiments performed with nuclear extracts from these cells fail to detect the proteins found in E7 QNR nuclear extracts (30).

We recently identified within the *Pax-QNR* gene a second promoter, termed P1, located 3 kbp downstream from the P0 promoter (32). However, the 3′ neuroretina-specific enhancer described in this study has no activity on this promoter. Since the TATA box sequences of the two responsive promoters (P0 and TK) are identical (ATATTAAGG) and differ from those of the P1 promoter (ATAAAGCAATAT), it was tempting to test whether the specificity of the enhancer for the P0 promoter and its failure to interact with the P1 promoter were attributable to the divergent TATA box sequences of the two promoters, as has been described for the myoglobin gene (45). However, when the P0 TATA box sequence was changed to that of the P1 promoter, the responsiveness of the enhancer was not abolished (data not shown). Thus, the differential in enhancer activities between P0 and P1 promoters may reside in other *cis*-acting promoter sequences. Alternatively, since recent studies suggest that some enhancer factors are dependent upon auxiliary factors (coactivators) for activation functions (for a review, see reference 39), it remains possible that the enhancer is unable to activate the P1 promoter because this promoter lacks its full complement of required factors to interact with the active enhancer. Another explanation is the presence of an inhibiting factor that prevents the P1 promoter from interacting with the proteins of the active enhancer. These hypotheses about enhancer mechanisms could also be invoked in the differential in enhancer activities observed in the neuroretina between E5 and E7. In E5 neuroretina, the enhancer could be inactivated by a repressor or the enhancer activity needs the presence of an activator, absent at this stage. Cloning the gene(s) encoding the enhancer element-binding factor(s) and analyzing the molecular interactions between the enhancer element-binding factor(s) and other transcription factors will allow us to further define precise molecular mechanisms involved in the regulation of the *Pax-6* gene.

Preliminary results suggests that there may be another important *cis*-acting element(s) within the promoter-distal region that also contribute to the neuroretina-specific expression of the *Pax-QNR* gene. Defining additional regulatory elements that control the tissue-specific developmental expression of the *Pax-6* gene may be essential for in vivo studies of the role of this gene in normal eye development and for determining the mechanisms of pathogenesis associated with *Pax-6*. Indeed, it remains possible that mutations in the *Pax-6* regulatory elements cause developmental defects associated with this gene, as has been suggested for some aniridia cases showing no mutations in the *Pax-6* coding region (18, 40). Although point mutations in the regulatory sequences associated with pathogenesis are rare, this has been demonstrated for the β-globin cluster (4).

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