Nicotinic acetylcholine receptors (nAchRs) mediate amacrine to ganglion cell synaptic transmission in the developing mammalian retina. The clustered neuronal nAchRs subunit genes, α3 and β4, are expressed in amacrine and ganglion cells where they are used to assemble functional receptor subtypes. The transcriptional mechanisms underlying expression of these subunits in retina are not yet known but may involve enhancers that are selectively active in retinal neurons. We previously identified a neuron-selective enhancer, β43′, whose activity in neural cell lines is dependent on ETS domain-binding sites. To determine whether β43′ is active in retinal neurons that express the α3 and β4 genes, we investigated β43′ activity in primary dissociated rat retinal cultures. We found that β43′ is selectively active in retinal neurons compared with retinal non-neuronal cells. This activity was derived primarily from amacrine and ganglion neurons, which are the retinal neuron cell types that express the clustered genes. Moreover, β43′ was selectively active in retinal neurons compared with cerebral cortical neurons suggesting that it is not a pan-neuronal enhancer. ETS factor-binding sites in the enhancer are required for its retinal neuron activity. These findings suggest that ETS factor interactions with β43′ control retinal neuron expression of certain nAchR subtypes.

Nicotinic acetylcholine receptors (nAchRs) are a family of ligand-gated ion channels that are expressed in numerous central and peripheral neuron populations (1). In many instances, different neuronal populations express certain receptor subtypes that have distinct gating and channel properties as well as distinct physiological functions. Diverse gating and channel properties are produced through homomeric and heteromeric assembly of different receptor subunit combinations (2). Localization of nAchRs to either the postsynaptic, presynaptic, or preterminal subcellular compartments is a mechanism that confers distinct physiological roles for particular subtypes (3–5).

Several neuronal nAchR subunit genes are expressed in the retina including the α3 and β4 genes, which are clustered in the genome, but not α5, the other member of the cluster (6–12). The α3 and β4 subunits are likely to be assembled into at least one kind of retinal neuron nAchR subtype (13, 14). Moreover, recent evidence (15–17) has implicated an α3-containing receptor in propagation of spontaneous action potential waves that are important for establishing patterns of retinal neuron synaptic connections. Before a neuron can assemble and assign a specific physiological function to a particular nAchR subtype, however, the appropriate combination of subunit mRNAs must be expressed in that cell. In contrast to the growing understanding of the expression and function of nAchRs in retinal neurons, nothing is known about the transcriptional mechanisms that direct expression of α3 and β4 genes to these cells.

The α3 gene promoter does not appear to contain cell type-specific information, which has led us to hypothesize that the clustered nAchR genes are under transcription control of neuron-selective enhancers (18). In support of this hypothesis, we identified an enhancer located in the β43′ untranslated region about 2.5 kb upstream of the α3 gene, which is currently the only known enhancer element in the cluster (19). Two distinct cis elements within the β43′ enhancer that bind ETS domain factors are required for its activity in neural cell lines (18). Interest in this enhancer as an essential component of regulatory information in the cluster arises from its differential activity in different cell types. First, it displays neural cell type-specific activity in a manner that correlates well with expression of the endogenous clustered genes in cell lines. Second, transfection experiments in dissociated peripheral primary neuron/non-neuronal cell cultures have shown that expression of reporter genes that are controlled by β43′ is largely limited to neurons (18). These characteristics support the idea that ETS domain factor interactions with β43′ are important for neuron-selective transcriptional control of one or more genes in the cluster.

Because the enhancer is located between the α3 and β4 promoters and the α3 and β4 genes are expressed in retina, β43′ may control retinal neuron expression of these genes. However, it is not yet known whether β43′ is active in retinal neurons. Here we have transfected dissociated rat primary neuron/non-neuronal retinal cell cultures with luciferase reporters to determine whether β43′ is active in retinal neurons. The data presented suggest that β43′ is a retinal neuron enhancer that may control expression of the cluster genes in these cells through interactions with ETS domain factors.

EXPERIMENTAL PROCEDURES

Plasmids

The pGL3 vector series (Promega Corp., Madison, WI) was used to prepare luciferase reporter constructs. The rat β43′ (1–90) sequences and mutations or deletions of it were prepared using three-way ligations of vector and synthetic oligonucleotides as described (18). The
AdMLP construct contains sequences –55/+10 of the adenovirus 2 major late promoter cloned upstream of the luciferase reporter. β43’-ETS Site Activity in Retinal Neurons

β43’- ETS Site Activity in Retinal Neurons

Retinal Cultures—Retinas were dissected from P1 Sprague-Dawley rat pups (Zivic Miller, Portersville, PA) and dissociated in dispase/collagenase or 5 mg/ml dispase alone (in the course of these experiments it was found that cell viability was much better when dissociation was carried out with dispase alone) for 12–15 min. Following a rinse in serum-containing medium, retinas were triturated with a fire-polished Pasteur pipette in serum-containing medium with 3.5% bovine serum albumin (Invitrogen), and 5 × 10^5 cells were plated into each well of poly-L-lysine-coated 24-well plates or onto 12-mm glass coverslips (Fisher). Cells for 6–8 wells of a 24-well plate were typically obtained from each animal (two retinas). Cultures were allowed to grow for 3 or 7 days before transfection, with fresh media added every 3 days.

β43’ reporters prepared using synthetic oligonucleotides were confirmed by dyeo sequencing using Sequenase reagents according to the manufacturer’s protocol (Amersham Biosciences); all other constructs were confirmed by restriction digests. Oligonucleotides were synthesized by Invitrogen, and plasmids for transfections were prepared using Qiagen reagents (Qiagen, Santa Clarita, CA). At least two different plasmid preparations were used for each construct, and all constructs were tested in at least three independent experiments.

Cell Culture and Transfection

 Luciferase Assays

Luciferase assays were carried out according to the manufacturer’s protocol (Promega Corp., Madison, WI) 24 h after transfection. Cells were lysed in 75 μl of lysis buffer per well of 24-well plates, and 10 μl of the lysate were assayed in 50 μl of luciferin substrate using a Lumat LB9501 luminometer (EG & G Berthold, Nashua, NH).

Immunocytochemistry and Immunohistochemistry

For ICC, cells were fixed for 25–30 min at room temperature with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. ICC was carried out as described (22) using the following antibody dilutions: rabbit anti-luciferase (Accurate Chemicals, Westminster, NY) 1:1000; mouse anti-βIII tubulin (Sigma) 1:3000; monoclonal antibody VC1.1 (Sigma) 1:3000; rabbit anti-L1 (gift of V. Lemmon, Case Western Reserve University) 1:1000; mouse anti-calbindin (Sigma) 1:1000; undiluted mouse anti-Thy-1.1 hybridoma supernatant (gift of A. Hall, Case Western Reserve University); fluoroconjugated goat anti-rabbit IgG and rhodamine isothiocyanate-conjugated goat anti-mouse IgG (Cappel, Durham, NC) 1:200. Coverslips were mounted in PBS/glycerol.

All ICC/IHC data were analyzed on a Nikon microscope. Cell counts were performed directly from immunostained photographs. Photomicrographs were digitally processed from color slides or black and white prints using Adobe Photoshop.

RNAse Protection

RNA was isolated from retinal cultures grown 4 days in serum-free medium using RNazol (Tel-Test, Friendsville, TX); 10–20 μg of RNA was used for each protection reaction. PC12 cell RNA was used as a positive control, and Rat2 RNA was used as a negative control for both Pet-1 and o3 (23). RNA protection was carried out using the RPAII kit (Ambion, Dallas Center, IA) according to the manufacturer’s instructions. The o3 (24) and Pet-1 (23) probes were prepared as described, including gel purification.

RT-PCR

RNA was isolated from retinal cultures as described above. Reverse transcription was carried out with 1–4 μg of Moloney murine leukemia virus reverse transcriptase (Invitrogen) and 35 cycles of PCR at 60 (ERM, ER81) or 55 °C (PEA3) using a PerkinElmer Life Sciences thermal cycler. Primers were constructed from the mouse sequences as follows: ERM, 5’-TCT AGA GAT GGG TTT TGT CAG CAA –3’ and 5’–GGT ACC GTA AGC GAA GCC TTC GGT GAT-3’ (primers described in Ref. 25; amplify nt 13–1539 of mERM); ER81, 5’-CGA CGA GCT CAT GGA TGG ATT TTA TGA CCA G-3 (amplify nt 1631 of mER81 (26)); PEA3, 5’-CAG TTC TCT TAG ACA CCC TTC TTC AGC AAA TCT CCC GG-3’ and 5’-CAG TGA GTC CGG ACC AGG CCT CCA GAA CTT GGT G3’ (amplify nt 397–899 of murine PEA3 (25)).

Retinal Nuclear Extracts

Mini-nuclear extracts were prepared from retinal cultures that had been grown in vitro for 4 days. Cells were lysed in 75 μl of lysis buffer (10 mM HEPES, pH 7.9; 10% glycerol; 10 mM KCl; 8% glycerol) was incubated with competitors, in 1.5 μl of lysis buffer per well of 24-well plates, and 10 μl of the lysate were assayed in 50 μl of luciferin substrate using a Lumat LB9501 luminometer (EG & G Berthold, Nashua, NH).

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For ICC, cells were fixed for 25–30 min at room temperature with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. ICC was carried out as described (22) using the following antibody dilutions: rabbit anti-luciferase (Accurate Chemicals, Westminster, NY) 1:1000; mouse anti-βIII tubulin (Sigma) 1:3000; monoclonal antibody VC1.1 (Sigma) 1:3000; rabbit anti-L1 (gift of V. Lemmon, Case Western Reserve University) 1:1000; mouse anti-calbindin (Sigma) 1:1000; undiluted mouse anti-Thy-1.1 hybridoma supernatant (gift of A. Hall, Case Western Reserve University); fluoroconjugated goat anti-rabbit IgG and rhodamine isothiocyanate-conjugated goat anti-mouse IgG (Cappel, Durham, NC) 1:200. Coverslips were mounted in PBS/glycerol.

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RNA was isolated from retinal cultures as described above. Reverse transcription was carried out with 1–4 μg of Moloney murine leukemia virus reverse transcriptase (Invitrogen) and 35 cycles of PCR at 60 (ERM, ER81) or 55 °C (PEA3) using a PerkinElmer Life Sciences thermal cycler. Primers were constructed from the mouse sequences as follows: ERM, 5’-TCT AGA GAT GGG TTT TGT CAG CAA –3’ and 5’–GGT ACC GTA AGC GAA GCC TTC GGT GAT-3’ (primers described in Ref. 25; amplify nt 13–1539 of mERM); ER81, 5’-CGA CGA GCT CAT GGA TGG ATT TTA TGA CCA G-3 (amplify nt 1631 of mER81 (26)); PEA3, 5’-CAG TTC TCT TAG ACA CCC TTC TTC AGC AAA TCT CCC GG-3’ and 5’-CAG TGA GTC CGG ACC AGG CCT CCA GAA CTT GGT G3’ (amplify nt 397–899 of murine PEA3 (25)).

Retinal Nuclear Extracts

Mini-nuclear extracts were prepared from retinal cultures that had been grown in vitro for 4 days. Cells were lysed in 75 μl of lysis buffer (10 mM HEPES, pH 7.9; 10% glycerol; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM dithiothreitol; 0.5 mM phenylmethylsulfonyl fluoride; 4 μg/ml leupeptin; 1 μg/ml apro tin; 1 μg/ml pepstatin). Nonidet P-40 (Roche Molecular Biochemicals) final concentration 0.5% was added immediately, and the cells were mixed by inverting several times. Nuclei were pelleted by brief centrifugation at 4 °C and extracted with Buffer C (20 mM HEPES, pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM dithiothreitol; protease inhibitors). Extracts were microcentrifuged at 4 °C for 10 min, and the supernatant was saved. Protein concentrations were determined using the BCA assay (Bio-Rad) and were typically in the range of 10 μg/ml. Cells were then lysed in 75 μl of 1× TGE buffer (75 mM Tris, 570 mM glycerin, 3 mM EDTA), 33 mM KCl, 8% glycerol, poly(dI-dC) (1 μg/reaction), and protease inhibitors for 30 min on ice. After addition of 0.1 μl of probe (5–10 × 10^6 cpm), reactions were further incubated for 15 min at 37 °C. Before loading, 10 μl of 1× TGE, 10% glycerol were added.
Retinal Neuron Cultures Express the a3 Gene—To confirm that a3-expressing neurons were present in primary retinal cultures, RNase protection was carried out with RNA from cultures grown 4 days in vitro. a3 mRNA was clearly detected in RNA samples obtained from several different independent cultures, consistent with the presence of retinal ganglion and amacrine cells (Fig. 1).

β43′ Directs Neuron-selective Gene Expression in Retinal Cultures—By having confirmed that retinal cultures are a valid system in which to assay transcriptional elements present in the neuronal nAChR gene cluster, we set out to investigate whether β43′ is active in retinal neurons. Dissociated retinal cultures were composed of about 10% neuron-specific tubulin-immunoreactive neurons after 3 days in vitro and only 2–3% after 7 days in vitro because of extensive non-neuronal cell proliferation. The majority of the non-neuronal cells were immunoreactive for glial fibrillary acidic protein, suggesting the presence of Müller glial cells, although a small number of Thy-1-positive fibroblasts were also present (data not shown). When retinal cultures were transfected after 3 days in vitro with a luciferase reporter carrying β43′-(1–90) upstream of the a3 promoter (β43′/a3), nearly 100% of the luciferase-expressing cells were neurons (Fig. 2, A, B, and E, and Table I). The same result was obtained with β43′ in reverse orientation (Fig. 2E and Table I). To demonstrate that non-neuronal cells can be efficiently transfected in these cultures, we scored luciferase-positive neurons and non-neuronal cells after transfection of a luciferase reporter carrying the SV40 promoter and SV40 enhancer (SV40E/SV40P). In clear contrast to that found for β43′/a3, approximately equal numbers of neurons and non-neuronal cells were luciferase-positive over the course of 15 independent experiments with the SV40E/SV40P reporter (Fig. 2, C, D, and E). The difference in the percentage of neurons expressing the luciferase reporter driven by β43′ versus SV40E was highly significant (Table I). Because the ratio of luciferase-positive neurons and non-neuronal cells is ~1:1 in SV40E/SV40P transfections, rather than the 1:9 that would be expected based on the composition of the cultures, it is possible that SV40E/SV40P also has a cell type-specific bias toward expression in neurons. However, we cannot distinguish this possibility from an alternative explanation, which is a bias toward transfection of neurons in these experiments. Nevertheless, the finding that both neurons and non-neuronal cells express the SV40 reporter demonstrates that both types of cells were transfected. Thus, the highly significant difference in cell type-specific activity between SV40E/SV40P and β43′/a3 supports the idea that the β43′ enhancer is a transcriptional element that together with the a3 promoter is able to drive retinal neuron-specific gene expression.

To determine whether the a3 promoter is required for neuron-selective expression in retinal cultures, assays were performed with reporters, β43′/SV40P and β43′/AdMLP, in which the a3 promoter was replaced with either the adenoviral major late (AdMLP) or the simian virus 40 (SV40) promoters. Both of these promoters are essentially TATA boxes and therefore are not expected to direct cell type-specific transcription on their own. In cultures transfected after 3 days in vitro with either reporter, the majority of the luciferase-expressing cells were neurons regardless of β43′ orientation (Fig. 2E and Table I). When retinal cultures were transfected with a reporter carrying the a3 promoter and SV40 enhancer (SV40E/a3P), more non-neuronal cells than neurons expressed detectable luciferase, similar to what was seen with SV40E/SV40P (Fig. 2E, Table I). This further confirms that both neurons and non-neuronal cells were being transfected but that β43′-driven reporters are preferentially expressed in neurons. Thus, the neuron-selective activity of β43′ does not require the a3 pro-
moter, and this promoter is not sufficient to direct a neuronal pattern of gene expression in retinal cultures. We conclude that the β43' enhancer is both necessary and sufficient to drive neuron-selective expression in these cultures.

We next investigated whether β43' neuron selectivity is maintained under conditions in which the ratio of non-neuronal cells to neurons is increased in cultures. To test this, we transfected cultures with the various reporters after 7 days in vitro; this incidentally resulted in higher transfection efficiencies (compare luciferase-positive cells in Tables I and II). As before, neither SV40E/SV40P nor SV40E/α3P supported neuron-selective expression of luciferase. In contrast, when β43'/α3P was transfected ~90% of the luciferase-expressing cells were neurons regardless of β43' orientation (Fig. 3 and Table II). Thus neuron selectivity was maintained even when neurons constituted only a small percentage of the cells in culture. Significantly more neurons were luciferase-positive in cultures transfected with either β43'/SV40P or β43'/AdMLP than with SV40P/SV40E. The percentage of luciferase-positive cells that were neurons, however, was lower in transfections with β43'/SV40P or β43'/AdMLP than in cultures transfected with β43'/α3P (Fig. 3 and Table II); this difference was statistically significant in some cases. These results suggest that the discrimination of β43' between neurons and non-neuronal cells may be supported by its natural promoter.

β43' could achieve neuron selectivity by silencing promoter activity in non-neuronal cells. To test this idea, we transfected retinal cultures with reporters driven by both the β43' and SV40 enhancers and the SV40 promoter. If β43' silences promoter activity in non-neuronal cells, this construct should have similar neuron specificity to reporters driven by β43' alone. We found, however, that this reporter is highly expressed in both neurons and non-neuronal cells (Table II), suggesting β43' does not silence promoter activity in non-neuronal cells.

**β43' Activity in Retinal Ganglion and Amacrine Neuron Cell Types—**Both retinal ganglion cells and amacrine cells in the ganglion cell and inner nuclear layers of the retina express α3 and β4 mRNA from about E13 into maturity (7–9, 12). To determine whether β43'-driven reporters are also expressed in amacrine and ganglion cells, we quantitated colocalization of retinal cell type markers with β43'-driven GFP. Two retinal ganglion cell markers were used, L1 and Thy-1. The localization of β43'-driven GFP to ganglion cells was first tested by immunostaining against the cell adhesion molecule L1, which is specific for ganglion cells in the retina (28). L1 immunoreactivity was largely restricted to neurites and growth cones, but processes that were positive for both L1 and β43'-driven GFP could be clearly identified (Fig. 4, A and B). Because of the density of L1-expressing neuritic processes, and the length of the processes, we were unable to quantify L1-GFP colocalization, which would require identification of the cell body producing the neurite. Thy-1 has been used extensively for the identification of retinal ganglion cells (29). We found that 45–55% of the neurons expressing β43'-driven GFP could be identified as retinal ganglion cells by Thy-1 expression (Fig. 4, C and D). Together, these data are consistent with the L1 colocalization and support the conclusion that a large proportion of the neurons expressing β43'-driven GFP are retinal ganglion cells.

Amacrine-specific markers were not available, but the monoclonal antibody VC1.1 recognizes an epitope present on horizontal and amacrine cells, but not retinal ganglion cells, in the retina (30). Many VC1.1 immunoreactive neurons were observed in our retinal cultures. Approximately 30% of the β43'-driven GFP-expressing neurons were immunoreactive for VC1.1 (Fig. 4, E and F). The number of horizontal cells in the mammalian retina is far smaller than the number of amacrine cells (31), which suggests that the majority of VC1.1-positive GFP-expressing cells are amacrine. Consistent with this, calbindin immunoreactivity, a marker for horizontal cells (30, 32), was detected in about 1% of GFP-expressing cells (Fig. 4, G and H). Together, these results suggest that at least 75–85% of the neurons expressing the β43'/α3P-GFP reporter were ganglion (Thy-1+) and amacrine (VC1.1+, Calbindin-) cells. The remaining neurons are likely to be bipolar neurons or ganglion cells that were undetected due to the weak immunoreactivity observed with Thy-1. Although occasional transfected photoreceptors were observed in retinal cultures, these cells have distinct morphologies and were not included in the analysis of retinal neuron subtype markers.

**Magnitude of β43' Transcriptional Activation in Different Neuronal Cell Types—**As an additional measure of the neuron-selective activity of β43', we quantitated the magnitude of β43'-enhanced luciferase expression in either dissociated retinal or cerebral cortical cultures. We compared the ratio of β43'/α3P-driven luciferase activity to α3-driven luciferase activity across cultures. In the conditions used here for retinal and cortical cultures, most of the transfected cells were neurons (see “Experimental Procedures”). Therefore, luciferase activity is derived primarily from neurons, and as presented in

### Table I

| Reporter       | No. neurons | No non-neuronal cells | Sum | n | Average % neurons ± S.E. | p value vs. SV40E/SV40P | p value vs. β43'/α3P |
|----------------|-------------|-----------------------|-----|---|-------------------------|-------------------------|----------------------|
| SV40E/SV40P    | 292         | 151                   | 443 | 15| 47 ± 8                  | <0.0001                 | <0.0001              |
| β43'/α3P       | 1201        | 27                    | 1228| 14| 97 ± 1                  | <0.0001                 | 0.98                 |
| β43'/AdMLP     | 1108        | 80                    | 1188| 12| 97 ± 1                  | <0.0001                 | 0.98                 |
| β43'/SV40P     | 993         | 18                    | 1011| 9 | 92 ± 3                  | <0.0001                 | 0.63                 |
| rSV40P/α3P     | 293         | 5                     | 298 | 10| 99 ± 1                  | <0.0001                 | 0.53                 |
| SV40E/α3P      | 56          | 85                    | 141 | 16| 37 ± 9                  | 0.25                    | <0.0001              |

**Fig. 3. Retinal neuron selectivity of β43' is maintained at higher gfp to neuron ratios.** The indicated reporters were transfected into dissociated retinal cultures, maintained for 7 days in vitro, and assayed after 30 h. Neuron selectivity was determined from counts of immunostained cultures for each of several experiments (Table II), and the percentages were averaged. Error bars are S.E.


Figs. 2–5, this activity is primarily from ganglion and amacrine cells in retinal culture transfections. Quantitation of luciferase activity indicated that the enhancer increased α3P reporter gene expression in cerebral cortical cultures, which contain many different types of neurons, by only a few fold. A small number of cortical neurons express α3, and therefore β43' activity observed in cortical cultures could arise from a small number of neurons in which the enhancer is highly active, or a low level of activity in many different types of neurons. In contrast, a 25-fold mean stimulation was detected in dissociated retinal cultures isolated from P1 rats (Fig. 5). In some experiments the stimulation was as high as 60-fold. This comparison indicates that β43' is not equally active in different types of neurons, and therefore β43' is not likely to be a pan-neuronal enhancer.

ETS Domain-binding Sites in β43' Are Required for Its Retinal Neuron Activity—The data presented in Figs. 1–5 and Tables I and II support the hypothesis that β43' is a retinal neuron enhancer involved in controlling retinal neuron-specific nAChR gene expression. This raises the question of what elements within the enhancer are important for its activity and what transcription factors interact with these elements. Analysis of various fragments and point mutations of β43' in PC12 cells has suggested that it is composed of at least two interacting cis elements that can bind ETS domain factors (18). The enhancer is much stronger in retinal neurons than in either PC12 cells (18) or cortical neurons (Fig. 5). Therefore, we investigated whether the same cis elements are responsible for enhancer activity in retinal neurons or whether additional β43' elements that are silent in PC12 cells and cortical neurons augment enhancer activity in retinal neurons. We first prepared various deletions of the enhancer through either of its two repeats while leaving the 6-bp spacer region intact (Fig. 6). These were then tested for stimulation of reporter activity in the context of the AdMLP after transfection into retinal cultures. Neither of the isolated repeats increased reporter activity to more than 60-fold levels, but deletions as small as 45 bp caused the enhancer activity in retinal neurons to be reduced to about 50% (Fig. 6, B). These results are similar to those obtained in the PC12 cell line, and therefore, it is not likely that the enhancer uses additional cis elements to stimulate high levels of transcription in retinal neurons.

To determine whether the ETS domain-binding sites present in the enhancer are required for its activity in retinal neurons, we tested reporters carrying base substitutions in these sites.
To pursue further the possibility that complex A contains an ETS factor, a series of EMSAs were carried out using competitors that correspond to ETS sites characterized previously. Four ETS sites, which have been shown to interact with ETS2, GAPBα/PU1.1, PEA3, and ER81 (Table III), competed for complex A (Fig. 8D, lanes 4, 6, 8, and 10). However, oligonucleotides in which the GGA cores of three of the tested ETS sites were disrupted did not compete (Fig. 8D, lanes 5, 7, and 9). A different ETS site derived from the T-cell receptor α enhancer binds ETS-1 (34, 35) and competes for spacer ETS-binding proteins from PC12 cells (18). This site, however, did not compete for complex A (Fig. 8D, lanes 11 and 12). The ability of multiple ETS site sequences to compete for complex A and the dependence on the GGA core for competition strongly suggest that complex A contains an ETS factor. The finding that the T-cell receptor α site, which can bind ETS-1 (34), did not compete for complex A suggests that the ETS factor involved is more related to PEA3, GABPα, or PU1 than to ETS-1.

**DISCUSSION**

Elucidating the transcriptional mechanisms that underlie selective expression of certain genes in different neuronal cell types has the potential to help reveal how neuronal cell type diversity is generated in the nervous system. For example, the proper development and maintenance of cholinergic neurotransmitter systems depends on appropriate cell type-specific transcription of particular neuronal nAChR subunit genes in neurons. The clustered neuronal nAChR genes are expressed in numerous but far from all central and peripheral neuronal populations (11, 36–38). Moreover, the pattern of expression of each gene undergoes significant changes among these populations during embryonic development (12). Unfortunately, for most types of differentiated neurons, including cholinergic types, representative cell lines are not available for gene transcription studies. Primary neural cell culture offers an alternative approach that permits detailed molecular analyses of transcriptional cis elements in normal neurons (18, 39). By using primary neural cell cultures that express the clustered nAChR subunit genes, we have investigated the cell type-specific activity and cis elements of the nAChR β43′ enhancer. The main findings presented are as follows. 1) The β43′ enhancer is selectively active in retinal neurons compared with non-neuronal cells and other kinds of central neurons. 2) The majority of retinal neurons expressing the β43′-driven reporter genes are amacrine and ganglion cells, which are the types of neurons that express the clustered nAChR subunit genes. 3) Retinal neuron activity of the enhancer depends on at least two different ETS domain-binding sites. 4) In contrast to many other cis elements identified in neuronal genes (40–44), β43′ does not appear to use negative elements to direct retinal neuron-selective expression. These results are discussed in terms of a model in which ETS domain binding to β43′ regulates transcription of one or both of the a3 or β4 genes as a first step in controlling expression of certain neuronal nAChR subtypes at cholinergic synapses of the retina.

**Selective Activity of β43′ in Retinal Neurons**—To begin to understand transcriptional control within the cluster, much attention has focused on the promoter regions of these genes. A common feature of these promoters across species is the presence of several Sp1 sites embedded in a G + G-rich region that initiates transcription at numerous nucleotides (45–50). Thus, it is not surprising that the differential activity of these promoters in different cell lines does not display a strict concordance with expression of the endogenous clustered genes in these lines. These characteristics make it difficult to account for the complex and dynamic neuronal expression patterns of the clustered nAChR genes solely through promoter-directed
transcriptional events and have led us to hypothesize that enhancers are operating in the cluster to influence promoter activity in certain neuronal populations.

The identification of the \( \beta \delta \epsilon \gamma ^{+} \) enhancer provides support for this idea because its activity among cell lines correlates well with expression of the endogenous clustered genes in these lines (19). Moreover, in physiologically more relevant primary neuron transfections, we have found that \( \beta \delta \epsilon \gamma ^{+} \) is selectively active in neurons versus non-neuronal cells (18), and as presented here it is strikingly more active in retinal than in cerebral cortical neurons. The weak activity of \( \beta \delta \epsilon \gamma ^{+} \) in cortical cultures is significant because it strongly suggests that \( \beta \delta \epsilon \gamma ^{+} \) is not a pan-neuronal enhancer that is equally active in all types of differentiated neurons. The data presented suggest instead that \( \beta \delta \epsilon \gamma ^{+} \) can direct two levels of cell type-selective transcription. First, it is largely inactive in non-neuronal cells. Second, it confers higher activity in only certain types of neurons.

Many vertebrate neuron-specific genes are regulated by cell type-specific silencers that repress their expression in non-neuronal cells (43, 51). In contrast, several lines of evidence suggest that \( \beta \delta \epsilon \gamma ^{+} \) does not use silencers to direct neuron-selective expression. First, sequences similar to the neuron-restrictive silencer element (43) and the GAP-43 repressor element (44) are not present in the enhancer. Second, a reporter containing both \( \beta \delta \epsilon \gamma ^{+} \) and SV40 enhancers and the SV40 promoter is weakly expressed in both neurons and non-neuronal cells of cerebral cortical neurons. The weak activity of \( \beta \delta \epsilon \gamma ^{+} \) in cortical cultures is significant because it strongly suggests that \( \beta \delta \epsilon \gamma ^{+} \) is not a pan-neuronal enhancer that is equally active in all types of differentiated neurons. The data presented suggest instead that \( \beta \delta \epsilon \gamma ^{+} \) can direct two levels of cell type-selective transcription. First, it is largely inactive in non-neuronal cells. Second, it confers higher activity in only certain types of neurons.

FIG. 7. Two different ETS domain-bindings sites are required for \( \beta \delta \epsilon \gamma ^{+} \) activity in retinal neurons. Transient transfections were performed in dissociated primary retinal cultures with the indicated reporters. The various reporters include either the intact \( \beta \delta \epsilon \gamma ^{+} \) enhancer (filled tandem rectangles linked by thin line) or modified enhancers (indicated by crosses) in which ETS-binding sites and other sequences were eliminated in either the first repeat alone, the second repeat alone, both repeats, or the spacer. Activity of the indicated reporters is presented relative to the activity of a reporter containing an intact enhancer, which is set at 100%. Also shown is the relative activity of the AdMLP in the absence of enhancer sequences. Bars represent the average % of \( \beta \delta \epsilon \gamma ^{+} \) (1–80) luciferase activity from at least three independent experiments \( \pm \) S.E. Asterisks indicate activities significantly different from \( \beta \delta \epsilon \gamma ^{+} \) (1–80) by analysis of variance.

\( \beta \delta \epsilon \gamma ^{+} \) ETS Site Activity in Retinal Neurons

ETS Factor Function in Retinal Neurons—Activity of \( \beta \delta \epsilon \gamma ^{+} \) in the PC12 neural cell line depends on two different ETS domain-binding sites. In addition, mobility shift assays with different ETS-binding site competitors and protein-DNA complex-blocking antibodies suggest that each of these sites binds different ETS domain factors expressed in this line (18). The results presented here show that these same ETS-binding sites are required for retinal neuron activity of \( \beta \delta \epsilon \gamma ^{+} \). Elimination of either repeat ETS site had little or no effect on \( \beta \delta \epsilon \gamma ^{+} \) activity, whereas elimination of both sites reduced enhancer activity to less than half of wild type. Significantly, elimination of the spacer ETS site nearly destroyed enhancer activity in retinal neurons. Together with activities determined for various \( \beta \delta \epsilon \gamma ^{+} \) fragments in retinal neurons, these results suggest that \( \beta \delta \epsilon \gamma ^{+} \) neuronal activity is the result of interactions among an ETS site in the spacer and redundant ETS sites in the repeats. As alluded to above, it is possible that additional undefined cis elements are present within the enhancer and are necessary along with the ETS sites for retinal neuron activity.

Our findings raise the question of which ETS domain factors are expressed in retinal amacrine and ganglion cells and which of these interact with the enhancer. There is currently little known about ETS domain gene expression in the vertebrate retina, and to our knowledge nothing is known about ETS factor function in this tissue. One study showed that the \( \text{erm}, \text{er81}, \text{and pea3} \) genes are expressed in the developing mouse neuroretina; however, whether these genes are expressed in amacrine or ganglion cells was not reported (25). mRNA for Pet-1, ERM, ER81, and PEA3 were detected in our cultures, suggesting they are candidates for regulating \( \beta \delta \epsilon \gamma ^{+} \). Our retinal cultures contain \( \beta \delta \epsilon \gamma ^{+} \)-binding proteins that require an intact ETS site for binding and are specifically competed by a variety of ETS sites. Together, these data are an indication, albeit indirect, that ETS domain factors may regulate gene expression in retinal neurons. An important future goal will be to

\[ \text{N. Francis and E. S. Deneris, unpublished observations.} \]
TABLE III

Oligonucleotides used for EMSA

| Oligo               | Sequence                                      |
|---------------------|-----------------------------------------------|
| R1                  | GGCTTATACAAAGGAAGTGCGATTGGGA                 |
| R1 ETSm             | GATCATGGGAAGGCTTATACAAATGTGGCATTGGAGCAGGC    |
| R2                  | TGCACTAGGAAGGCTTATACAAATGTGACCTGGAGGCACAG    |
| R2 ETSm             | TGCACTAGGAAGGCTTATACAAATGTGACCTGGAGGCACAG    |
| ETS2 (52)           | TCGACCAGGAAGTGACTTGCCGTTC                   |
| ETS2m               | TCGACCTAAAGTGACTtgaGGTC                     |
| PU.1/GABPα (26)     | GGCTTGGTGGGAAAGTATAAGAA                     |
| GABPα               | GGCTTGGTGGTGGAGTATAAAGAT                    |
| PEA3 (53)           | CCTGGCAAGGAAGTTCGA                         |
| PEA3m               | CCTGAGCTACGTGTCGA                           |
| ERS1 (26)           | TTATAGGGGGATGTTGCA                         |
| TCRα (35)           | CTCCTCTTCTCCAGAGAATATGTGGCAGCAGGA          |
| TCRαm               | CTCCTCTTCTCCAGATACGTGGCTTCTGCAGA           |

Fig. 8. Retinal cultures contain ETS transcripts and ETS-like β43'-binding proteins. A, Pet-1 mRNA is expressed in 4-day retinal cultures (4th lane). PC12 RNA (1st lane) was used as a positive control, and Rat2 fibroblast and yeast total RNAs (2nd and 3rd lanes) were used as negative controls. 20 μg of RNA was used for each RNase protection reaction, and the Pet-1-protected fragment is ~170 bases long. For PC12 RNA, 2 μg of RNA was mixed with 18 μg of yeast total RNA. B, mRNA for PEA3 family ETS factors ERM, ER81, and PEA3 are expressed in 4-day retinal cultures. Each set of three lanes consists of a reaction run without template (lanes 1, 4, and 7), without reverse transcriptase (lanes 2, 5, and 8), or with template and reverse transcriptase (lanes 3, 6, and 9). The expected sizes of the PCR products are 1.5 kb (ERM), 1.4 kb (ER81), and 0.6 kb (PEA3). Lane 10 is HindIII digested size markers. C, EMSAs (C and D) were carried out with a radiolabeled probe consisting of most of the R1 sequence (Table III), retinal nuclear extracts, and various oligonucleotide competitors. Several protein complexes were formed by retinal nuclear extracts on the R1 probe (lane 2) which were competed by an excess of cold R1 (lane 3) or R2 (lane 5). R1 or R2 with the ETS site mutated (Table III) did not compete effectively for complex A (lanes 4 and 6). Lane 1 shows probe incubated in the absence of extracts. Competitors were used at a 200-fold molar excess. D, EMSAs were carried out as in C with a variety of oligonucleotide competitors (Table III). Complex A was competed by wild type but not mutated ETS sites. Asterisks mark nonspecific complexes that were formed from certain batches of nuclear extracts. The molar excess of competitors used is as follows: lanes 2–5, 11, and 12 are ×200 and lanes 6–10 are ×400. The lanes shown are taken from several gels that have been aligned according to the position of complex A.
identify the ETS protein(s) present in complex A and determine their role in regulating nAchR gene expression.

What Is the Biological Role of β43′—nAchRs composed of β4 and α3 subunits are likely to function in the retina to mediate cholinergic synaptic transmission (13, 15). Assembly of these heteromeric nAchR subtypes, therefore, depends on coordinate regulation of the β4 and α3 genes, which suggest the existence of cis elements that are active in retinal neurons. β43′ is currently the only known transcriptional element in or near the clustered nAchR genes that is capable of supporting retinal neuron-selective gene transcription. This characteristic, together with the absence of α5 expression in retina, raises the possibility that ETS domain interactions with β43′ are required specifically for β4 or α3 expression in retinal ganglion and amacrine cells. The location of β43′ between the β4 and α3 promoters enables it to coordinate both genes in these neuronal cell types without influencing α5 transcription. In vivo molecular genetic approaches are ongoing to determine the biological function of β43′ and ETS factors in the vertebrate retina.

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REFERENCES

1. Sargent, P. B. (1993) Annu. Rev. Neurosci. 16, 403–443
2. McGee, D. S., and Role, L. W. (1995) Annu. Rev. Physiol. 57, 521–546
3. Jones, S., Sudweeks, S., and Yale, J. L. (1989) Trends Neurosci. 12, 555–561
4. McGee, D. S., and Role, L. W. (1996) Curr. Opin. Neurobiol. 6, 342–349
5. Wonnacott, S. (1997) Trends Neurosci. 20, 92–98
6. Britto, L. R., Rogers, S. W., Hamassaki-Britto, D. E., and Duvoisin, R. M. (1994) Visual Neurosci. 11, 569–577
7. Hamassaki-Britto, D. E., Brzozowska-Preciati, A., Karten, H. J., Lindstrom, J. M., and Keyser, K. T. (1991) J. Comp. Neurol. 313, 394–408
8. Hamassaki-Britto, D. E., Gardino, P. F., Hoke, J. N., Keyser, K. T., Karten, H. J., Lindstrom, J. M., and Britto, L. R. G. (1994) J. Comp. Neurol. 347, 161–170
9. Hoover, F., and Goldman, D. (1992) Exp. Eye Res. 54, 561–565
10. Keyser, K. T., Britto, L. R. G., Schoepfer, R., Whiting, P., Cooper, J., Conroy, W., Brzozowska-Preciati, A., Karten, H. J., and Lindstrom, J. (1993) J. Neurosci. 13, 442–454
11. Wada, E., Wada, K., Boulter, J., Deneris, E., Heinemann, S., Patrick, J., and Swanson, L. W. (1990) Brain Res. 526, 45–53
12. Matter-Sudzinski, L., Hernandez, M. C., Ritzel, T., Ballivet, M., and Matter, J. M. (1992) EMBO J. 11, 4529–4538
13. Jones, F. S., and Meech, R. (1999) Bioessays 21, 372–376
14. Kruman, S. D., Chong, J. A., Tsay, H.-J., and Mandel, G. (1992) Neuron 9, 37–44
15. Mori, N., Schoenherr, C., Vandenbergh, D. J., and Anderson, D. J. (1992) Neuron 9, 45–54
16. Schoenherr, C., Piquette, A. J., and Anderson, D. J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9881–9886
17. Weber, J. R. M., and Skene, J. H. P. (1997) J. Neurosci. 17, 7583–7593
18. Bigger, C., Casanova, E. A., and Gardner, P. D. (1996) J. Biol. Chem. 271, 32842–32849
19. Bigger, C. B., Melnikova, I. N., and Gardner, P. D. (1997) J. Biol. Chem. 272, 25976–25982
20. Campos-Caro, A., Carrasque-Serrano, C., Valor, L. M., Vineira, S., Ballesta, J. J., and Criado, M. (1999) J. Biol. Chem. 274, 4693–4701
21. Flora, A., Schulz, R., Benfante, R., Battaglioni, E., Terzano, S., Clementi, F., and Fornasari, D. (2000) J. Neurochem. 75, 18–27
22. Terzano, S., Flora, A., Clementi, F., and Fornasari, D. (2000) J. Biol. Chem. 275, 41495–41503
23. Yang, X., Fyodorov, D., and Deneris, E. S. (1995) J. Biol. Chem. 270, 8514–8520
24. Mandel, G., and McKinnon, D. (1993) Annu. Rev. Neurosci. 16, 323–345
25. Galang, C., K., Der, C. J., and Hauser, C. A. (1994) Oncogene, 9, 2913–2921
26. Xin, J.-H., Cowie, A., Lachance, J., and Hassell, J. A. (1992) Genes Dev. 6, 481–496

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