Functional Properties of the Neuronal Nicotinic Acetylcholine Receptor β3 Promoter in the Developing Central Nervous System

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Within the chick central nervous system, expression of the β3 nicotinic acetylcholine receptor gene is restricted to a subset of retinal neurons, the majority of which are ganglion cells. Transient transfection in retinal neurons and in neural and non-neural cells from other regions of the chick embryo allowed the identification of the cis-regulatory domain of the β3 gene. Within this domain, a 75-base pair fragment located immediately upstream of the transcription start site suffices to reproduce the neuron-specific expression pattern of β3. This fragment encompasses an E-box and a CAAT box, both of which are shown to be key positive regulatory elements of the β3 promoter. Co-transfection experiments into retinal, telencephalic, and tectal neurons with plasmid reporters of β3 promoter activity and a number of vectors expressing different neuronal (ASH-1, NeuroM, NeuroD, CTF-4) and non-neuronal (MyoD) basic helix-loop-helix transcription factors indicate that the cis-regulatory domain of β3 has the remarkable property of discriminating accurately between related members of the basic helix-loop-helix protein family. The sequence located immediately 3′ of the E-box participates in this selection, and the E-box acts in concert with the nearby CAAT box.

In vertebrates, both negative and positive regulations play an important role in determining neuronal gene expression. Negative regulation (reviewed in Refs. 1 and 2) is best exemplified by REST/NRSF, a factor that represses transcription of the SCG-10 and type II Na+ channel genes in non-neuronal cell-types, whereas most neurons lack REST/NRSF and thus express these two genes (3, 4). Conversely, the Olf-1 transcription factor positively regulates several genes (e.g. the olfactory neuron-specific G protein, the type III adenylyl cyclase) specifically expressed in olfactory neurons (5). Vertebrate homologs of the basic helix-loop-helix (bHLH) factors involved in Drosohila neurogenesis (6) act as positive or negative regulators in the acquisition of neuronal identity. For instance, the atonal- and achaete-scute-related activators are transiently expressed in parts of the central and peripheral nervous system during early development, and their null mutation or ectopic expression profoundly influences neurogenesis (reviewed by Lee et al. (7)). However, the direct regulation by bHLH proteins of genes that define neuronal identity has never been documented.

Several neuronal nicotinic acetylcholine receptor (nAChR) genes are expressed early in neural development (8–12), and, since they encode transmembrane sensors capable of fluxing Ca2+ and other cations upon stimulation (reviewed in Ref. 13), an understanding of their regulation should help explain how the genetic program puts together the mechanisms needed for epigenetic environmental cues to participate in development. This is illustrated in a recent report by Shatz and associates (14) showing that, in the developing retina, cholinergic synaptic transmission between newly generated amacrine and ganglion cells is responsible for the propagation of spontaneous waves of action potentials that may be critical for the establishment of visual system circuitry.

Several nAChR subunit genes, including α4, β2, β3, and β4, are expressed in the chick retina (10), and expression of β3 is confined to ganglion cells and amacrine neurons (15). Forsayeth and Kobrin (16) have shown that the β3 subunit co-assembles in vivo with the α4, β2, and β4 subunits to form a functional nicotinic receptor endowed with distinctive properties. We have isolated a short 5′-sequence of the β3 gene containing promoter elements that are sufficient to target reporter gene expression to those retinal neurons that normally express β3 in vivo (11, 15). The stringent neuronal specificity of the β3 promoter and its activation during the period of neuronal fate determination make it an attractive system in which to study the functional interactions between transcription factors and cis-acting regulatory elements that help establish the diverse neuronal phenotypes.

In this report, we carry out a functional analysis of the cis-regulatory domain, establishing that transcription of the β3 gene is under the direct control of bHLH factors. Moreover, we show that the β3 promoter is able to discriminate accurately between related members of the bHLH family, thereby effecting the stringent neuron-specific regulation of the gene.

EXPERIMENTAL PROCEDURES

DNA Constructions—Standard molecular biology techniques were used (17) unless otherwise stated. Construction of the reporter plasmids β3RS-CAT and β3RS-lacZ and of the reference plasmids SV-CAT and SV-lacZ was described by Hernandez et al. (15). Point mutations in the β3RS sequence (Fig. 1A) were introduced by polymerase chain reaction and checked by sequencing. The mutant DNA fragments were cloned at the Smal site of the pCAT00 plasmid (18). SF-E and SF-3E were obtained by ligation of PvuII linkers (CCAGCTGG; New England Biolabs) to the 5′-end of fragment SF (Fig. 2A). α1KK encompasses nucleotides 151–334 of the chick α1 nAChR promoter (19, 20) (GenBank accession number M15307), flanked by KpnI restriction sites that were used for subcloning into pCAT00. The α1β3 hybrid promoter was obtained by ligation of the 5′-end of α1KK with the 3′-end of β3RS at their shared PvuII restriction site (Fig. 7A). The expression plasmid

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The abbreviations used are: bHLH, basic helix-loop-helix; nAChR, nicotinic acetylcholine receptor; CAT, chloramphenicol acetyltransferase; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactosidase; CEF, chick embryonic fibroblast; E4–E13, embryonic days 4–13; bp, base pair(s); CNS, central nervous system.

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amount of acetylated 14C-labeled chloramphenicol was determined by transfection, cells were collected and processed for CAT assay. The grid areas that each contained about 10^3 positive cells upon transfection

Desoxyribonuclease I (Boehringer Mannheim) was added to 30 μl trypsin for 10 min (E4–E8) or with 0.1% trypsin for 20 min (E9–E13).

twice with ice-cold phosphate-buffered saline and lysed in guanidine thiocyanate (17). Total RNA was isolated, gel-fractionated, and hybridized as described (10, 15). We used as probe a 32P-labeled free probe; in A, F, bound probe.

RESULTS

Identification of Regulatory Elements in the β3 Promoter—In the chick CNS, the β3 nAChR gene is selectively expressed in the neuroretina. We have previously shown that essential neuron-specific promoter elements are located in a short EcoRI-Sphi I DNA fragment, 143 bp in length and located just upstream of the transcription start site (11, 15). This β3RS fragment contains several putative binding sites for transcription factors: CACCC, CAGCTG (E-box), CAAT, ATATA (TATA-like motif), or TCAAAA (AT-rich motif) as indicated (superscript). The arrow marks the transcription start site, and the dashed line identifies the wild-type and E-box mutant double-stranded oligonucleotides (35 bp) used in C. The β3RS promoter (143 bp) extends between the indicated EcoRI and Sphi I sites. B, the wild-type (E4–E8) and mutant sequences (*) were linked to the chloramphenicol acetyltransferase (CAT) gene. Cells isolated from E5 neuroretina were transfected with the constructs (5 μg) and assayed for CAT activity 48 h after transfection. The CAT activity obtained with the wild-type β3RS fragment is arbitrarily set at 100, and activities of the mutated promoters are given relative to this value. C, nuclear protein extracts prepared from E5 neuroretina and E9 optic tectum were used for gel mobility shift assays. The DNA binding affinity of these extracts (1, 3, or 5 μg of protein/assay) was tested on the wild-type (WT) or E-box mutant (E-box−) double-stranded 35-mer underlined in A, F. Free probe; B, bound probe.
bHLH Proteins Regulate the β3 nAChR Gene

Precise Positioning of the E-box and CAAT Box Is Required for Promoter Activity—Mutation of the CAAT box, which is located 9 bp downstream of the E-box, abolishes promoter activity in retinal cells (Fig. 1B), suggesting that direct interactions between proteins bound to these two elements may take place. Due to the helicity of DNA, the addition or deletion of base pairs in the intervening sequence should disrupt the alignment of bound factors, thereby decreasing promoter activity. Several mutants were constructed by the addition or deletion of nucleotides between the E-box and the CAAT box, and they were tested for promoter activity in E5 retinal cells (Fig. 3). Reducing the distance by 1, 2, and 3 bp was sufficient to decrease promoter activity 5, 8, and 10-fold, respectively. In contrast, the addition of 1–4 bp had either a modest effect (Fig. 3) or no effect at all (SF-E, SF-3E), depending on the particular additional base pairs. This suggests that steric hindrance between bound factors contributes to the decrease in promoter activity when the distance between the two motifs is reduced.

Functional Analyses of Neuronal bHLH Proteins—Since the E-box is a binding site for transcription factors of the bHLH family, we postulated that expression of the β3 gene is under the control of such a factor in the neuroretina. Several bHLH genes are sequentially expressed in the developing chick CNS. While CASH-1 is expressed in proliferating cells (26), NeuroM is transiently expressed in cells that have withdrawn from the

addition of one E-box restored promoter activity in retinal cells to a level comparable with that of the complete β3RS fragment, and three E-boxes allowed activity levels consistently higher than β3RS (Fig. 2, B and C). The tissue specificity of the reactivated promoters was examined by transfection into cells in which the β3 promoter is normally silent, namely cells from the optic tectum and telencephalon, CEFs or glial cells (Fig. 2C). No promoter activity was detected in any of them, indicating that SF-E and SF-3E are regulated as specifically as the β3RS fragment. Thus, the addition of one E-box is sufficient to reconstitute a promoter with the same specificity and activity as the wild-type fragment, demonstrating that the E-box is a key regulator of the β3 nAChR gene.

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mitotic cycle but have not yet migrated in the outer layers, whereas NeuroD labels neurons that are migrating and differentiating (21). CTF-4 (22) is widely distributed in the nervous system, but its expression is significantly enhanced in the retina (23). Since β3 expression at early stages of retina development coincides with expression of these different bHLH proteins, we tested if any of them was able to trans-activate the β3RS promoter. Co-transfections with expression vectors encoding CASH-1, NeuroM, NeuroD, or CTF-4 into E8 tectal and E9 telencephalic neurons revealed that none of these proteins was able to trans-activate the β3RS promoter ectopically, although all four were functional transcription factors capable of strongly enhancing activity of the E-box-driven promoter of the muscle nAChR α1 subunit (α1KK fragment, Fig. 4A, Table I).

Next, we tested whether overexpression of these neuronal transcription factors in retinal cells increased the activity of the β3 promoter. Cells isolated from E5 retinae were co-transfected with the β3RS-lacZ reporter and each of the CASH-1, NeuroM, NeuroD, or CTF-4 expression vectors. On its own, the β3RS promoter drives the expression of β-galactosidase in 15 ± 2% of E5 retinal cells, and this proportion remained unchanged in cells that overexpressed CASH-1, NeuroM, NeuroD, or CTF-4 (Table I). Moreover, no influence of these factors on β3 promoter activity has been detected at later stages of retina development (data not shown). The α1 nAChR gene is not expressed in retina, but the transfected α1KK fragment displayed a significant promoter activity in retinal cells (most likely due to transactivation by endogenous bHLH factors), and co-transfection of the α1KK-lacZ reporter with the different neuronal bHLH proteins strongly increased the proportion of X-gal-positive retinal cells (Table I). Thus, although CASH-1, NeuroM, NeuroD and CTF-4 are functional transcription factors and instances of overlapping expression between these neuronal bHLH genes and β3 in the retina have been detected, none of these factors appears to be directly involved in the regulation of β3. Gel mobility shift analyses reveal that CTF-4 binds to the β3 E-box in vitro (Fig. 4B) and that NeuroD binds to the CAGCTG motif (27). If such interactions take place in vivo, they are probably not sufficient for promoter activation. Weintraub et al. (28) have shown that the base pairs flanking one of the IgH E-boxes constitute part of a negative cis-acting element enabling the IgH gene to discriminate between bHLH proteins. In the case of β3, mutation of the base pairs flanking the E-box did not modify the promoter specificity toward neuronal bHLH proteins (Fig. 4C).

**Ectopic Activation of the β3 Promoter by a bHLH Protein—** Because functional domains are well conserved among members of the bHLH protein family, we asked if MyoD, a transcriptional activator of muscle-specific genes that binds to the CAGCTG motif (29), was capable of replacing endogenous neuronal bHLH proteins. Co-transfections with a MyoD expression vector revealed that ectopic expression of MyoD was sufficient to trans-activate the β3RS promoter in neurons from the optic tectum, telencephalon, and cerebellum that do not normally express β3 (Fig. 5, A and B). In parallel experiments, telencephalic or tectal cells were co-transfected with MyoD and β3RS-lacZ constructs, and trans-activation was observed by X-gal staining in about 20% of transfected cells (Table I). This activation is mediated by the E-box, since MyoD failed to activate the promoter bearing a mutant E-box (Fig. 5). Moreover, as determined by Northern blot analysis with RNA obtained from transfected tectal cells, MyoD was also able to trans-activate the endogenous β3 gene (Fig. 5C), clearly indicating that the transfected β3RS promoter behaves in neurons much as the native promoter does.

We also tested the ability of MyoD to trans-activate the β3RS promoter in non-neuronal cells. In cells from the pigment epithelium of the retina, MyoD was unable to trans-activate the β3RS promoter or the endogenous β3 gene (Fig. 5, A and C). We used the α1KK promoter to ascertain that functional MyoD was indeed synthesized in transfected pigmented retinal cells. Although the α1 nAChR gene is not expressed in this tissue, the transfected α1KK fragment displayed detectable promoter activity, which was strongly enhanced by ectopic expression of MyoD (Fig. 5A). Low levels of β3RS promoter activity were detected in glia selected from optic tectum or neuroretina or in embryonic fibroblasts (Fig. 5B). We believe that this trans-activation resulted from the presence of residual populations of

3 T. Roztocil, unpublished observation.

4 L. Matter-Sadzinski, M. Ballivet, and J.-M. Matter, manuscript in preparation.
neurons, since no X-gal-positive glial or fibroblast-like cells were detected when these cells were co-transfected with β3RS-lacZ and MyoD. Taken together, these results indicate that the β3 promoter is regulated by a bHLH protein that can be substituted by MyoD and that trans-activation of β3RS by MyoD is restricted to neurons, suggesting that additional neuron-specific co-activators are required. This view is further supported by the fact that there is no expression of the β3 gene in muscle (15) and no activity of the β3RS promoter in transfected myotubes (Fig. 7C), despite the presence there of MyoD and other myogenic bHLH factors.

Influence of MyoD on β3 Promoter Activity in the Developing Retina—We investigated the effect of MyoD on β3RS promoter activity within the domain of β3 expression. Transcription of β3 in neuroretina is first detected on E4, whereupon activity of the promoter rapidly increases and peaks on E5, decreasing later to relatively low levels (11). We wanted to determine whether ectopic MyoD could activate the β3 promoter earlier in development, increase the peak value at E5, or maintain a high level of activity at later stages. The MyoD expression vector was co-transfected with β3RS-CAT or with β3RS-lacZ into retinal cells isolated at different stages between E4 and E13. MyoD had no influence on promoter activity at early stages of development, but it enhanced CAT synthesis in the developed retina (E8–E13) without modifying the proportion of β-galactosidase-positive cells (Fig. 6; Table I). The levels of endogenous β3 mRNA were affected in the same way, with a MyoD-induced increase on E13 but not on E5 (data not shown). In contrast, MyoD strongly stimulated promoter activity of the α1KK fragment both in E5 and E13 retinal cells, indicating that functional MyoD was indeed synthesized in these cells (Table I). We interpret these observations as suggesting that endogenous neuronal bHLH protein(s) for which MyoD can substitute are not limiting in early retina, whereas later in development, ectopic expression of MyoD compensates for decreased amounts of the endogenous bHLH protein(s).

A Hybrid α1/β3 Promoter Behaves Like the β3 Promoter—Our experiments highlight the remarkable capacity of the β3RS promoter to discriminate between different members of the bHLH family, in striking contrast to the α1KK promoter, which was activated by all five of the bHLH proteins we tested. Because sequences in the vicinity of the E-box may play an important role in the recognition of a specific bHLH factor, we constructed a hybrid promoter from portions of the α1KK and β3RS fragments. The α1KK promoter contains two E-boxes. The distal α1 E-box and the β3 E-box have the same sequence (CACGTG), and we took advantage of the fact that it is a PvuII restriction enzyme recognition site to fuse the two promoters at this level (Fig. 7A). In the hybrid promoter, termed α1/β3, the 56 bp upstream of the reconstituted CACGTG E-box come from the α1 promoter, while the 69 bp downstream of it come from the β3 promoter. Promoter activity of the hybrid was compared with that of α1 and β3 in myotubes and in CEFs (Fig. 7C). As

| Cell types | Control | MyoD | NeuroD | CASH-1 | CTF-4 |
|------------|---------|------|--------|--------|-------|
| TelE9 1KK | α1      | 13 ± 3 (3) | 150 ± 10 (3) | 55 ± 5 (3) | 62 ± 5 (3) | 58 ± 5 (3) |
| β3       | 0 (6)   | 25 ± 5 (6)   | <1 (3)      | <1 (3)      | <1 (3)      |
| PE E5 β3 | 0 (3)   | 0 (2)        |            |            |            |
| NR E5 α1 | 37 ± 7 (4) | 120 ± 18 (4) | 81 ± 7 (4) | 70 ± 7 (4) | 78 ± 8 (4) |
| β3       | 15 ± 2 (20) | 15 ± 3 (5)   | 15 ± 3 (3) | 15 ± 3 (3) | 15 ± 3 (3) |
| NR E13 β3| 10 ± 2 (20) | 11 ± 3 (5)   |            |            |            |
FIG. 6. MyoD and β3 promoter activity in the developing neuroretina. The β3RS-CAT construct was co-transfected with either the MyoD (+) or the control expression vector (−) into retinal cells isolated from E4 to E13 (Hamburger and Hamilton stages 23–39; Ref. 25). Cells were assayed for CAT activity 24 h after transfection. The CAT activity obtained upon co-transfection with SV40-CAT plus the control expression vector is arbitrarily set at 100 for each developmental stage, and activities of the β3 promoter are given relative to this value.

A

B

C

expected, the α1 promoter had a strong activity in myotubes and was weak in CEFs. In contrast, the β3 and α1/β3 promoters were completely silent in both cell types. In co-transfections, MyoD consistently and strongly enhanced activity of the α1 promoter but did not influence the α1/β3 promoter. In telencephalic neurons, the β3 and α1/β3 promoters were completely silent and could not be trans-activated by CASH-1, NeuroM, NeuroD, or CTF-4 (Fig. 4 and data not shown). The hybrid promoter was only found to be active in retinal cells, where it reached an activity level somewhat lower than β3 (Fig. 7B). It is known from a previous study that the distal E-box of the α1 promoter is sufficient to drive reporter gene transcription in myotubes (30), yet when it is flanked in 3′ by sequences from the β3 promoter, its activity becomes restricted to retinal cells. Indeed, the hybrid promoter behaves much like the β3RS or SF-E fragments (Fig. 2), suggesting that the 69-bp sequence located downstream of the β3 E-box contains essential elements that act in concert with the E-box to confer stringent specificity upon the β3 promoter.

DISCUSSION

A short fragment, 75 bp in length and located immediately upstream of the transcription start site, is sufficient to generate the neuron-specific expression pattern of the β3 nAChR gene. Inquiring into the underlying mechanisms, we present evidence that the β3 promoter is positively regulated by an E-box acting in concert with a neighboring CAAT box. The β3 promoter appears to have a simple structure and be devoid of the regulatory complexities resulting either from inhibitory DNA elements that prevent expression in non-neuronal cell types (reviewed by Schoenherr and Anderson (2)) or from multiparite elements whose active combinations vary in the course of the development (31). Very few neuron-type specific promoters have been characterized in detail, and we do not know whether the simple organization of the β3 cis-regulatory domain is a common feature of genes whose expression is confined to restricted subsets of neurons.

The finding that an E-box is a key regulator of the β3 gene emphasizes the role of bHLH factors in neural transcriptional control. Several members of the bHLH family are transiently expressed in the developing CNS. ASH-1 is widely expressed in proliferating precursor cells (26). ASH-1 and neurogenin-1 (ATH-4C) exhibit complementary domains of expression in the neuroepithelium, suggesting that these early bHLH genes might be associated with specification of cell identity (32). The widespread expression of NeuroM and NeuroD in postmitotic cells at distinct times in neural development suggests that they do not define functionally distinct neuronal phenotypes but rather successive stages of a cell’s life course (21, 33). Transcription of the β3 gene is activated in a small subset of proliferating retinal cells, and then it is continuously expressed during cell differentiation and in the mature ganglion and amacrine cells (11). Although ASH-1, NeuroM, and NeuroD are expressed in subsets of retinal cells and instances of overlapping expression between these factors and β3 have been detected, we found that the β3 promoter was also active in cells that do not express these bHLH genes. Co-transfection experiments have confirmed that the ASH-1, NeuroM, and NeuroD proteins do not control transcription of the β3 gene. None of the known neuronal bHLH proteins is present throughout the period of β3 expression, and, although we cannot rule out the possibility that β3 is sequentially regulated by distinct bHLH proteins during development, we favor the idea that it is regulated by a particular, unidentified bHLH protein whose expression is associated with specific neuronal phenotypes.

Our results demonstrate that MyoD is able, upon ectopic expression in central neurons, to induce transcription of both transfected and endogenous β3 promoters. MyoD itself is absent from the nervous system, but expression in the developing brain of several other myogenic regulatory genes such as mef-2 and myf-5 suggests that interesting parallels may exist between muscle and neuron differentiation (34, 35). Although MyoD acts as an activator of β3 in subsets of central neurons, it is incapable of inducing transcription of this gene in non-
neuronal cells, suggesting that induction requires additional co-activators that are exclusively expressed in neurons. In early retina, MyoD has no influence on β3 promoter activity, presumably because the bHLH protein, which controls β3 transcription and for which MyoD can substitute, is available in nonlimiting amounts. Since ectopic expression of MyoD does not increase the proportion (about 15%) of retinal cells expressing β3, we propose that the endogenous bHLH protein must, like MyoD, synergize with neuronal co-activators that, in the retina, are confined to the subset of neurons forming the domain of β3 expression. However, these or similar co-activators are also present in other subsets of neurons elsewhere in the developing CNS, as demonstrated by MyoD's ability to trans-activate β3 in a fraction of neurons from different regions of the CNS (Table I). Thus, β3 expression depends on the presence in the same neuron of both the appropriate bHLH protein and the appropriate co-activators. In the retina, such a combination is predicted to occur in ganglion and amacrine cells.

The ability of the β3 promoter to distinguish between different members of the bHLH family determines its stringent neuron-type specificity. The mechanism by which E-boxes discriminate in vivo between related bHLH proteins is poorly understood. We have shown that although CTF-4 binds to the β3 E-box with high affinity in vitro, it is not competent to activate β3 transcription in vivo. Our experiments did not provide evidence that the base pairs flanking the β3 E-box constitute part of a negative cis-acting element that may prevent ASH-1, NeuroD, NeuroM, and CTF-4 from functioning as co-activators. In the retina, such a combination is predicted to occur in ganglion and amacrine cells.

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