Sp1 and Sp3 Regulate Expression of the Neuronal Nicotinic Acetylcholine Receptor β4 Subunit Gene*

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Neuronal nicotinic acetylcholine receptors play important roles in signal transduction within the nervous system. The receptors exist in a variety of functionally distinct subtypes that are determined by their subunit structures. The subunits are encoded by 11 genes, α2–α9 and β2–β4. Three of the genes, α3, α5, and β4, are tightly clustered, and their encoded proteins make up the predominant receptor subtype in the peripheral nervous system. The tight linkage of the genes suggests there may be a common regulatory mechanism underlying their expression. However, although their expression patterns significantly overlap, they are not identical, indicating that independent regulatory mechanisms must also exist. Our studies have focused upon the gene encoding the β4 subunit for which we have identified several transcriptional regulatory elements. One of these elements, E2, specifically interacts with the general transcription factor Sp1. Here we show that another member of the Sp family of factors, Sp3, can specifically interact with E2 whereas two other members, Sp2 and Sp4, cannot. Co-transfection experiments indicate that Sp3 can transactivate a β4 promoter/reporter gene construct and, furthermore, that Sp1 and Sp3 can transactivate the β4 reporter construct synergistically. The transactivation is dependent upon an intact E2 and may involve direct interactions between Sp1 and Sp3.

Ligand-gated ion channels are critical signaling components of the nervous system. They are required for generating the electrical signals of neurons that underlie information transmission within the nervous system. The most extensively studied ion channels are nicotinic acetylcholine (nACh) receptors which are encoded by a family of at least 16 genes (1, 2). Eleven of these genes (α2–α9 and β2–β4) are expressed within neuronal populations and can form functionally distinct heteromeric and homomeric receptor subtypes (3–5). The functional diversity exhibited by the neuronal nACh receptor family results from the differential expression and incorporation of the different subunits into mature receptors. We would like to understand the molecular events leading to formation of the various receptor subtypes. It is clear that regulation, both positive and negative, at the level of transcription plays a key role in the establishment of the differential expression patterns of the subunit genes (6–19). Consequently, our laboratory has focused upon characterizing the transcriptional regulatory mechanisms involved in the expression of a cluster of receptor subunit genes, those encoding the α3, α5, and β4 subunits. This cluster of genes spans approximately 60 kilobase pairs of the rat genome (20). As the α3, α5, and β4 subunits make up the predominant nACh receptor subtype expressed in the peripheral nervous system (21), the clustering of their genes raises interesting possibilities regarding regulation of their expression. One possibility is that they are coordinately expressed via a common regulatory mechanism. However, although the genes exhibit overlapping expression patterns, the expression patterns are not completely identical (5, 22–27) suggesting that in addition to a possible common regulatory mechanism, these genes are most likely subject to independent regulatory mechanisms as well.

As an initial approach to understanding the regulation of the clustered receptor subunit genes, we have focused upon the gene encoding the β4 subunit. We previously reported the identification of several regulatory elements within the promoter region of the β4 gene and demonstrated that these elements specifically interact with nuclear proteins present in extracts prepared from brain tissue and a cholinergic (used here to describe cells that either synthesize and release acetylcholine and/or are cholinoceptive) cell line (11, 16). One of these elements, E1, was shown to interact with the transcriptional regulatory factor Puro, as well as three other, unidentified, DNA-binding proteins that we refer to as neuronal acetylcholine receptor promoter-binding proteins (Ref. 28). Another element, E2, was shown to interact with the general transcription factor, Sp1 (16). We further demonstrated, via transient transfection analyses, that Sp1 is capable of transactivating a β4 promoter/reporter gene construct (16). These results, and others, led us to hypothesize that the E1-binding proteins facilitate or strengthen interactions between E2 and Sp1 leading ultimately to β4 gene expression (16).

Sp1 was originally identified as a cellular transcription factor required for SV40 gene expression (29, 30). Subsequently, Sp1 was shown to activate transcription of a wide variety of cellular and viral genes (30–33). More recently, a family of Sp1-related factors has been identified and includes Sp2, Sp3, and Sp4 (34–36). Sp1, Sp3, and Sp4 recognize GC and CACCC boxes with similar specificities and affinities, which is reflected in the high degree of conservation between their DNA-binding domains (34, 35, 37). In contrast, Sp2 binds GC boxes with a significantly lower affinity than the other Sp family members,
pointing to the complexity of gene regulation by the Sp family (36). This complexity is further illustrated by the demonstration that Sp1 and Sp4 function as transcriptional activators, whereas Sp3 functions as a transcriptional activator or repressor depending upon the gene in question (35, 38).

Given the interaction between E2 of the β4 subunit gene and Sp1, we investigated whether other Sp family members interact with E2. In this report, we show that Sp3 can interact specifically with E2, that in co-transfection experiments Sp3 can transactivate a β4 promoter/reporter gene construct, and that Sp1 and Sp3 can transactivate a β4 promoter/reporter gene construct in a synergistic manner. In addition, we demonstrate that Sp1 and Sp3 are expressed in cholinergic cells. Finally, we present data suggesting that the transactivation by Sp1 and Sp3 is a consequence of the two factors interacting.

**EXPERIMENTAL PROCEDURES**

**Electrophoretic Mobility Shift Assays—**Electrophoretic mobility shift assays (EMSA) were performed as described (11) with minor modifications. A radiolabeled double-stranded DNA oligonucleotide was incubated in the presence of nuclear extract, prepared from either SN17 cells (39) or adult rat brains, in the presence of nonspecific competitor poly(dC:dC). The oligonucleotide corresponded to either a β4 regulatory region (sequence shown in Fig. 1) or the consensus Sp1 binding site (5′-ATTCCATGGGGGCGGCG-GAGC-3′). Following the addition of the radiolabeled oligonucleotide, the reactions were incubated on ice for 45 min. The reactions were then separated through 6.5% polyacrylamide gels (prerun at 100 V for 30 min) in 0.5× TBE buffer (150 mM Tris-HCl, 39 mM glycine, 20% methanol, 0.037% SDS) for 1 h at 100 V. Membranes were blocked in Blotto (5% nonfat dry milk in Tris-buffered saline) for 1 h at room temperature. Western blotting with anti-Sp1, anti-Sp2, and anti-Sp3 was performed using the SuperSignal Western System (Pierce) according to the manufacturer’s instructions. Anti-Sp1, anti-Sp2, and anti-Sp3 antibodies were used at concentrations of 1, 5, and 5 μg/ml, respectively, in Blotto solution.

**Immunoprecipitations and Western Blotting—**SN17 cells were grown in 150-mm dishes and harvested at 70% confluency. Immunoprecipitations and Western blotting were performed as described previously (41). Briefly, cells were washed twice with PBS, scraped in PBS, and pelleted. Cell pellets were resuspended in 500 μl of cell lysis buffer (1% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 100 μg/ml aprotinin in PBS) and lysed on ice for 30 min. Nuclei were lysed by passage through a 21-gauge needle 5 times, and cell debris was removed by centrifugation. Immunoprecipitations of cell extracts were performed for 1 h at 4 °C with gentle rolling with 8 μl of either anti-Sp1 or anti-Sp3 rabbit antisera. Immune complexes were recovered on protein G-agarose beads (Santa Cruz Biotechnology, Inc.) for 1 h at 4 °C with gentle rolling. The beads were pelleted, washed 4 times with 1 ml of 1% Nonidet P-40 in PBS, and resuspended in 30 μl of Laemmli SDS loading buffer (2% SDS, 100 mM dithiothreitol, 50 mM Tris-HCl, pH 6.8, 10% glycerol, 0.1% bromophenol blue). Samples were boiled for 5 min, and 10 μl of each sample was electrophoresed through SDS-12% PAGE. Western blot analysis with either anti-Sp1 or anti-Sp3 antibody was performed as described above.

**RESULTS**

**Plasmids and Transfections—**Drosophila Schneider SL2 cells were obtained from the American Type Culture Collection and were maintained in room temperature in modified Schneider’s Drosophila medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and antibiotics. Cells were seeded at 2 × 10⁵ per 60-mm culture dish immediately prior to transfection. DNAs were introduced into the cells by liposome-mediated transfection using Perfect Lipids kit (Invitrogen) as recommended by the manufacturer. Transfections in SN17 cells were performed using Pfx-6 lipid from the kit (Tropix).

**Expression constructs containing Sp1 or Sp3 coding sequences were transcribed and translated in the presence of 35S-labeled methionine using the TNT-Coupled Reticulocyte Lysate System (Promega Corp.) according to the manufacturer’s instructions. Proteins translated in vitro (20 μl) were incubated with antibodies against either anti-Sp1 or anti-Sp3 rabbit polyclonal antibody in 150 μl of cell lysate buffer (see above). Immunoprecipitations were carried out as described in the preceding paragraph. Following electrophoresis, gels were treated with Enhance (NEN Life Science Products), dried, and subjected to autoradiography.

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Regulation of Acetylcholine Receptor Gene Expression

or anti-Sp4 antibodies did not affect the EMSA pattern (Fig. 2A, lanes 3, 5, 9, and 11), whereas incubation with anti-Sp1 and anti-Sp3 antibodies led to the appearance of at least one additional complex (complex A) having a lower mobility than either complex B or C (Fig. 2A, lanes 2, 4, 8, and 10). Interestingly, incubation of anti-Sp1 with the binding reaction led to a greater decrease in complex B as compared with complex C, whereas incubation of anti-Sp3 in the reaction had the opposite effect, there being a greater decrease in complex C as compared with complex B. Incubation of the binding reaction with both antisera simultaneously led to an almost complete absence of both complexes B and C with a concomitant increase in complex A (i.e. it appeared to have a synergistic effect; Fig. 2A, lanes 6 and 12). The negative results obtained with the anti-Sp2 and anti-Sp4 antibodies raise the question as to whether the two factors are present within the samples tested and, if so, whether the antibodies can recognize them. To address this question, mobility supershift experiments were carried out using a consensus Sp1-binding site as the probe and the same anti-Sp2 and anti-Sp4 antibodies used in the experiments with the E1/E2 oligonucleotide. Incubation of the radiolabeled consensus Sp1-binding site with nuclear extract alone led to the formation of two protein-DNA complexes (Fig. 2B, complexes B and C). Incubation of the binding reactions with anti-Sp4 antibody led to the appearance of an additional complex having a lower mobility than either complex B or C (Fig. 2B, complex A, lanes 3 and 6). Incubation of the binding reaction with anti-Sp2 antibody did not affect the EMSA pattern (Fig. 2B, lanes 2 and 5). Although these data indicate that Sp4 is present in the SN17 and rat brain extracts and that the anti-Sp4 antibody can recognize it, the question remains regarding the presence of Sp2. To investigate whether Sp2 is present in SN17 cells and rat brain, Western blot analysis was carried out using extracts from SN17 cells and rat brain. As shown in Fig. 2C, two bands of approximately 85 and 80 kDa were observed when the blot was incubated with the anti-Sp2 antibody, consistent with the published size of Sp2 (36) and indicating that Sp2 is present in the extracts at a concentration sufficient for detection by the antibody. These results are consistent with previously published data which suggest that the DNA binding specificity of Sp2 is different from those of the other Sp factors (36). Taken together, these data indicate that Sp1 and Sp3 or antigenically related proteins, but not Sp2 or Sp4, can interact with the β4 regulatory region.

FIG. 1. The promoter region of the rat nACh receptor β4 subunit gene. The positions of two transcriptional regulatory elements, E1 and E2, within the β4 5′-flanking region and the DNA sequence encompassing the elements are shown. The oligonucleotide used in EMSA is indicated as E1/E2 oligo. pX1B4FHwt denotes a luciferase expression vector containing the FokI to HindIII fragment as indicated. pX1B4FHmut4 is a luciferase expression vector in which E2 is mutated as indicated by the underlines (see Ref. 16).

FIG. 2. Sp1 and Sp3, or antigenically related proteins, interact with the E1/E2 region. A, EMSA were performed using SN17 and rat brain nuclear extracts. End-labeled E1/E2 oligonucleotide (50 fmol) was incubated with 12 μg of the indicated extract alone (lanes 1 and 7), in the presence of anti-Sp1 antisera (lanes 2 and 8), anti-Sp2 antisera (lanes 3 and 9), anti-Sp3 antisera (lanes 4 and 10), anti-Sp4 antisera (lanes 5 and 11), or both anti-Sp1 and anti-Sp3 antisera (lanes 6 and 12). Complexes are indicated by lettered arrows with complexes B and C being the previously reported specific complexes (16) that are supershifted (complex A) upon incubation with antisera specific for Sp1, Sp3, or both. B, EMSA were performed using SN17 and rat brain nuclear extracts. End-labeled Sp1-binding site oligonucleotide (50 fmol) was incubated with 12 μg of the indicated extract alone (lanes 1 and 4), in the presence of anti-Sp2 antisera (lanes 2 and 5), or anti-Sp4 antisera (lanes 3 and 6). Complexes are indicated by lettered arrows with complexes B and C being the previously reported specific complexes (16) that are supershifted (complex A) upon incubation with antisera specific for Sp4. C, Western blot analysis was carried out with three amounts (2, 5, and 10 μg) of SN17 whole cell extract, three amounts (10, 20, and 40 μg) of rat brain nuclear extract, and anti-Sp2 antisera. Numbered arrows indicate the sizes (in kDa) of the two Sp2 species.

Sp1 and Sp3 Transactivate β4 Promoter Activity in a Synergistic Manner—Given the results of the mobility supershift experiment, the functional relevance of Sp1 and Sp3 was tested. Drosophila Schneider SL2 cells (which have been reported to lack Sp activity; Refs. 31, 32, 35, and 42) were transfected with a wild type β4 promoter/luciferase expression construct (pX1B4FHwt; see Fig. 1) and an expression construct for either Sp1 (pActSp1; Ref. 40) or Sp3 (pPacUSp3; Ref. 35). To confirm that Sp1 or Sp3 expression was involved in transacti-
Sp1 and Sp3 transactivate, in a synergistic manner, a β4 promoter/luciferase construct in Drosophila cells. Drosophila SL2 cells were co-transfected with either pX1B4FHwt (left panel) or pX1B4FHmut4 (right panel). In the case of pX1B4FHwt, cells were co-transfected with the pX1B4FHwt alone, with increasing amounts of an Sp1 or Sp3 expression construct separately and together, or with increasing amounts of both Sp1/3 expression vectors devoid of Sp1/3 coding sequences (labeled as vectors). Luciferase values were normalized to β-galactosidase expression as driven by a Rous sarcoma viral promoter. Fold induction was calculated relative to the normalized luciferase activity obtained by transflecting pX1B4FHwt alone. With regard to pX1B4FHmut4, fold induction was calculated relative to the normalized luciferase activity obtained by transflecting pX1B4FHmut4 alone. Transfections and normalizations were done as for pX1B4FHwt.

When 10 fmol of each Sp expression construct were used, Sp1 transactivated the promoter approximately 200-fold (Fig. 3). The residual activation is seen with the mutated promoter may be a consequence of the Sp factors on the transcriptional activity of the mutated β4 promoter/luciferase construct (Fig. 3). When Sp1 and Sp3 were both co-transfected with the β4 promoter/luciferase construct, an approximately 200-fold increase in transcriptional activity was seen, indicating a synergistic effect of the two regulatory factors (Fig. 3). In addition, transactivation of the β4 promoter/luciferase construct by Sp1 and Sp3 occurs in a dose-dependent manner. Ten fmol of each Sp expression construct were used, Sp1 transactivated the β4 promoter approximately 30-fold while Sp3 transactivated the promoter approximately 200-fold (Fig. 3). Ten fmol of both Sp expression constructs co-transfected with the wild type β4 promoter led to an approximately 280-fold activation (Fig. 3). To determine the dependence of the transactivations on an intact E2, similar transfection experiments were carried out using a β4 promoter/luciferase construct in which E2 is mutated at three nucleotide positions (pX1B4FHmut4; see Fig. 1). The effects of the Sp factors on the transcriptional activity of the mutated β4 promoter were significantly less than those on the wild type promoter, there being only an approximately 60-fold activation when 10 fmol of both Sp expression constructs were used (Fig. 3). The residual activation seen with the mutated promoter may be a consequence of the Sp factors binding to other sites within the promoter (see “Discussion”).

To determine whether the Sp factors had any effect on β4 promoter activity in a more physiological context, a series of experiments was carried out using the SN17 cholinergic cell line. First, to determine whether the SN17 cells do in fact express Sp1 and Sp3, Western blot analysis was performed. As shown in Fig. 4, a single band of approximately 97 kDa was seen when the blot was incubated with anti-Sp1 antiserum, consistent with the published size of Sp1 (43). When anti-Sp3 antiserum was used, two bands of approximately 98 and 60 kDa were observed (Fig. 4A), again consistent with the previously reported sizes of Sp3 (43). Second, co-transfection experiments were carried out using the SN17 cell line. The effects seen with Sp1 and Sp3, either separately or together, were much less than those seen in Drosophila cells (Fig. 4C). Only a 2.7-fold induction was seen when both Sp family members were co-transfected with the wild type β4 promoter construct. This is in contrast to the 280-fold induction seen in Drosophila cells (Fig. 3). One possible explanation for these results is that the endogenous levels of the Sp factors may be so high that exogenously added Sp factors (as expressed from transfected plasmids) may not alter the overall concentration of the factors significantly, and therefore, any effects would be greatly diminished or even absent. To test this hypothesis, Western blot analysis was carried out using extracts prepared from SN17 cells transfected in parallel with the SN17 cells used for reporter gene assays (Fig. 4C). As shown in Fig. 4, D and E, the levels of Sp1 and Sp3 in transfected cells are comparable to those in mock-transfected cells, indicating that transfection of the Sp expression constructs does not appreciably alter the intracellular concentrations of the factors. Thus, it seems likely that the endogenous Sp levels in SN17 cells are so high as to preclude any effects from the transfected factors. These observations are consistent with published reports using other mammalian cell lines (35, 44).

Sp1 and Sp3 May Directly Interact—The mobility supershift and transfection experiments strongly implicate both Sp1 and Sp3 in the transcriptional regulation of the β4 subunit gene. Furthermore, it appears that they transactivate the β4 promoter by binding to the same site, E2. The question arises as to whether one of the Sp factors binds preferentially to E2 and then the other factor exerts its effects on β4 promoter activity via protein-protein interactions. Alternatively, it is possible that both factors bind simultaneously to E2 resulting in transcriptional regulation. As a first approach to this question, immunoprecipitations followed by Western blot analysis were performed. Anti-Sp1 or anti-Sp3 antisera was used to immunoprecipitate proteins from an SN17 extract. The precipitated proteins were separated via SDS-PAGE and blotted onto a nitrocellulose membrane that was subsequently incubated with anti-Sp3 or anti-Sp1 antiserum. Two specific bands were seen when proteins from SN17 cell lysates were precipitated with an anti-Sp1 antiserum and then probed with an anti-Sp3 antiserum on a Western blot (Fig. 5A). The sizes of the bands, 98 and 60 kDa, correspond to the published sizes of Sp3 (43). In the reciprocal experiment, a protein of approximately 97 kDa that reacts with an anti-Sp1 antiserum on a Western blot was precipitated from SN17 cell lysates with an anti-Sp3 antibody (Fig. 5A). Preimmune rabbit serum did not precipitate either
Sp1 or Sp3 in this experiment (Fig. 5). To confirm the specificities of the antibodies, immunoprecipitations were carried out using in vitro translated 35S-labeled Sp1 and Sp3. As shown in Fig. 5B, there appears to be no cross-reactivity between the two antibodies with anti-Sp1 precipitating Sp1 but not Sp3 and anti-Sp3 precipitating Sp3 but not Sp1. These data provide compelling evidence for direct physical interactions between Sp1 and Sp3, leading to significant implications regarding the mechanism by which Sp1 and Sp3 may regulate β4 subunit gene expression.

**DISCUSSION**

The mechanisms regulating transcription of neuronal nACh receptor subunit genes remain to be completely elucidated. However, considerable effort has led to the identification of a number of cis-acting regulatory elements (6, 16, 18, 19, 46–48), and the proteins interacting with these elements are beginning to be characterized (9, 11, 12, 16, 17, 28, 46, 49). With respect to the β4/α3/α5 gene cluster in rat, the majority of what is known regarding transcriptional regulation pertains to the α3 and β4 subunit genes. Deneris and colleagues (17, 46) have shown that the POU domain factor SCIP/Tst-1 is capable of activating the α3 subunit promoter in vitro and that this activation is dependent upon the POU domain of SCIP/Tst-1. The activation by SCIP/Tst-1 occurs independently of the SCIP/Tst-1 binding sites located in the promoter region of the α3 gene raising the possibility that the transcriptional effect of SCIP/Tst-1 is a consequence of protein-protein interactions perhaps involving the basal transcriptional machinery, as previously suggested (46). Interestingly, another subfamily of

**FIG. 4.** Sp1 and Sp3 are highly expressed in the SN17 cholinergic cell line, precluding any effects of exogenously added Sp1 or Sp3. Western blot analysis was carried out with three amounts (2, 5, and 10 µl) of SN17 extract and anti-Sp1 antiserum (A) and anti-Sp3 antiserum (B). Arrows indicate the sizes (in kDa) of molecular mass markers. A, 5 ng of purified Sp1 were included on the blot as a positive control. C, SN17 cells were transiently transfected with pX1B4FHwt and 670 fmol of an Sp1 or Sp3 expression construct separately and together or with 670 fmol of both Sp1/3 expression vectors devoid of Sp1/3 coding sequences, as indicated. Luciferase values were normalized to β-galactosidase expression as driven by a Rous sarcoma viral promoter. Fold induction was calculated relative to the normalized luciferase activity obtained by transfecting pX1B4FHwt alone. D and E, Western blot analysis of mock-transfected SN17 cells (SN17) and SN17 cells transfected with expression vectors for either Sp1 (D) or Sp3 (E). Three amounts (2, 5, and 10 µl) of SN17 extract were used in each case. D, the blot was incubated with anti-Sp1 antiserum, and in E the blot was incubated with anti-Sp3 antiserum. Arrows indicate the sizes (in kDa) of molecular mass markers.

**FIG. 5.** Immunoprecipitations of Sp1-Sp3 complexes from SN17 cells lysates. A, anti-Sp1 antiserum, anti-Sp3 antiserum, or preimmune rabbit serum were used to immunoprecipitate (IP) proteins from an SN17 extract. Western analysis of the immunoprecipitated material was carried out using anti-Sp1 or anti-Sp3 antiserum as indicated. The asterisk indicates a nonspecific signal. Numbered arrows indicate the sizes of molecular mass markers. B, the anti-Sp1 and anti-Sp3 antisera do not cross-react in immunoprecipitations. The first two lanes are in vitro translated 35S-labeled Sp1 and Sp3. Anti-Sp1 and anti-Sp3 antisera were used to precipitate the in vitro translated 35S-labeled Sp1 and Sp3, as indicated for the next four lanes. Arrows indicate specific in vitro translated products.
POU domain factors, the Brn-3 subfamily, has been shown to differentially regulate α3 subunit promoter activity in vitro; Brn-3a activates the α3 promoter whereas Brn-3b and Brn-3c repress α3 promoter activity (49). The Brn-3 POU transcription factors have no effect on β4 subunit gene expression (49). Deneris and colleagues (18) have also identified a PC12 cell-specific enhancer, termed β43′, in the 3′-untranslated exon of the β4 subunit gene which forms PC12 cell-specific DNA-protein complexes in EMSA. β43′ is an apparently novel regulatory element composed of two nearly identical 37-base pair repeats separated by 6 base pairs (18). The enhancer is capable of activating transcription from a variety of promoters including the α3 and β4 subunit promoters (18). The physiological relevance of β43′ remains to be determined, although initial transgenic studies utilizing mice carrying a 2.8-kilobase pair fragment of the β4/α3 intergenic region, which includes β43′, suggest it may be important for establishing part of the restricted pattern of α3 gene expression in the central nervous system (18), but it is clearly not sufficient for conferring reporter gene expression in the peripheral nervous system where the α3 subunit, in combination with the α5 and β4 subunits, makes up the predominant nACh receptor subtype (21).

The emerging picture, then, is that Sp3 is a bifunctional protein factor family and the rat β4 subunit gene promoter. We previously demonstrated that Sp1 can induce β4 promoter activity in a Drosophila cell line approximately 10-fold (16). The data presented in this report indicate that in addition to Sp1 one other Sp family member, Sp3, can significantly induce β4 promoter activity. Neither Sp2 nor Sp4 were shown to interact with E2 as judged by EMSA and, thus, were not pursued in terms of functional relevance. The inductive effects of Sp3 were approximately 10-fold higher than those of Sp1 in Drosophila SL2 cells. Interestingly, co-transfection experiments indicated that the transcriptional effects of Sp1 and Sp3 are synergistic suggesting that the two factors can simultaneously bind and activate the β4 promoter. The transcriptional activation of Sp1 and Sp3 is dependent upon an intact E2 as transfection experiments with a mutated E2 led to much reduced effects of the regulatory factors. However, there was still a 60-fold induction of the mutated promoter at the highest concentrations of Sp1 and Sp3 expression constructs used. It is likely that the residual activation is a result of the factors binding to other consensuses Sp-binding sites, of which there are two located downstream of E2 (16). Although the roles these additional sites play in β4 gene expression are unknown, it is important to note that they are low affinity binding sites for the Sp factors.

The demonstration that Sp3 can function as an activator of transcription is in contrast to earlier reports that indicated Sp3 is an inhibitory transcriptional factor (35, 44, 51–53). More recently, however, a number of studies indicated that Sp3 can also function as a positive regulatory factor (38, 43, 50). The emerging picture, then, is that Sp3 is a bifunctional protein. In strong support of this hypothesis is a recent study demonstrating that Sp3 contains domains that can both activate and repress transcription and that the predominant Sp3 function is dependent upon both the promoter and the cellular context (38). The molecular mechanism underlying the dual function character of Sp3 remains to be elucidated. It has been suggested that Sp3 might functionally interact with the components of the basal transcriptional machinery, presumably as part of the TFIIID complex, since co-transfection of Sp3 with TATA-binding protein resulted in super-activation of a reporter gene (38). Given the structural and functional similarities between Sp1 and Sp3, the interaction between Sp3 and TATA-binding protein may occur through formation of protein-protein contacts in a manner analogous to Sp1 and involving the TATA-binding protein-associated factor dTAF110 (54, 55).

Potential functional interactions between Sp1 and Sp3 with the β4 promoter in cholinergic cells were difficult to ascertain as the endogenous factors are expressed at relatively high levels. That the effect of simultaneously transfecting Sp1 and Sp3 expression constructs led to only an approximately 2-fold induction over control values of β4 promoter activity, is most likely a consequence of the inability to significantly alter, by overexpression, the intracellular concentrations of the factors. However, data from immunoprecipitation/Western blot analyses strongly suggest that Sp1 and Sp3 may directly interact. Taken together, the data presented in this study indicate that Sp1 and Sp3 can bind and activate the β4 subunit promoter at E2 simultaneously in a synergistic manner. We previously hypothesized that the proteins binding to E1 and E2, which are adjacent to each other (see Fig. 1), may interact to regulate β4 gene expression (16). We recently biochemically purified from bovine brain four proteins that bind to E1. Amino acid sequence analysis indicated that one of the proteins is the transcriptional regulatory factor Purα (28). The identities of the three other proteins remain to be determined. Preliminary in vitro binding assays indicate that Sp1 and Purα directly interact (potential Sp3/Purα interactions are currently being investigated). This observation and the immunoprecipitation/Western blot data presented above suggest a model in which Sp1 and Sp3 bind E2 and Purα binds E1 with Sp1 participating in protein-protein interactions with both Sp3 and Purα (Fig. 6). How these protein-DNA and protein-protein interactions are ultimately coupled to the RNA polymerase II machinery to regulate β4 gene expression remains to be determined. In this regard, it is important to note that the β4 promoter does not contain a TATA sequence (10), and thus, it is possible that Sp3 (and perhaps Sp1) is linked to components of the basal transcriptional complex via interactions with tethering factors as has been postulated for Sp1 activation of TATA-less promoters.

2 I. N. Melnikova, unpublished observations.
(Fig 6; Refs. 33 and 56) or via interactions with the TFID complex (as discussed above). How the other three E1-binding proteins participate in β4 gene regulation awaits their identification, but they presumably interact directly with Pur-β as they were co-purified with Pur-β (28).

Acknowledgments—We thank Drs. Edward Seto, Tom Shenk, and Guntram Suske for Sp1 and Sp3 expression constructs, and Dr. Steve Britt, Elizabeth Casanova, and Quin Du for useful discussions and technical advice.

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