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The POU Domain of SCIP/Tst-1/Oct-6 Is Sufficient for Activation of an Acetylcholine Receptor Promoter

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In the PC12 neuroendocrine line, the neuronal nicotinic acetylcholine receptor α3 gene promoter is activated by SCIP/Tst-1/Oct-6, a POU domain transcription factor proposed to be important for regulating the development of specific neural cell populations. In this study, we have investigated the SCIP polypeptide domains involved in α3 promoter activation. The characteristics of activation by a chimeric effector in which the GAL4 DNA binding domain was substituted for the SCIP POU domain were dramatically different from those of wild-type SCIP. At low effector masses, the chimeric polypeptide weakly activated α3 in a GAL4 binding-site-dependent manner but then squelched transcription at higher masses. In contrast, wild-type SCIP activation was not modulated by the presence of multimerized SCIP binding sites, and squelching was not observed. Analysis of wild-type SCIP truncations revealed that deletion of the previously characterized SCIP amino-terminal activation domain did not destroy activity of the factor. Surprisingly, a truncation expressing nothing more than the POU domain was nearly as active as wild-type SCIP. Moreover, cotransfection of a GAL4-VP16 effector with an effector expressing just the SCIP POU domain resulted in synergistic activation of the promoter. Synergistic activation did not depend on an Sp1 motif that is the only functional α3 *cis* element outside the transcription start site region. Our results show that the DNA binding domain of a POU factor is capable of transcriptional activation probably through protein-protein interactions with components of the basal transcription complex.

POU domain factors constitute a large subfamily of homeodomain proteins that have been implicated in the control of cell commitment, proliferation and differentiation (35, 47, 48, 52). The POU domain within each of these factors is composed of two subdomains, the POU-specific domain, which is unique to these proteins, and the POU homeodomain, which is distantly related to the archetypal *Drosophila* homeodomain (14). The two POU subdomains are tethered by a short, variable-length, unstructured linker to form a highly conserved bipartite DNA binding domain and an interface for specific protein-protein interactions with other POU factors as well as unrelated transcription factors (18, 33, 41, 42, 43, 49, 50). These proteins can either activate or repress gene transcription, depending on the particular POU factor, target sequence, and cell context (reviewed in references 48 and 52). In virtually all reported instances, POU domain action has been shown to be dependent on high-affinity binding of the factors to specific A+T-rich sequence motifs positioned either within or at a variable distance from promoter elements. For example, cell-type-specific activation of the growth hormone and prolactin genes is dependent on high-affinity binding of Pit-1 to several related A+T-rich sequence motifs positioned upstream of their promoters (2, 15, 31). Similarly, various POU factors, including Oct-1 and Oct-2, can transactivate genes via the A+T-rich octamer motif (17, 28, 29, 37). This motif is important for B-lymphocyte-specific transcription of immunoglobulin genes (19, 20, 54) as well as transcription of many other genes that are expressed in either a cell-specific or ubiquitous manner (reviewed in reference 48). More recently identified POU factors are also capable of transactivating promoters in an octamer-dependent manner (22, 38, 44). Some of these, however, transactivate by binding to A+T-rich elements that are distinct from octamer motifs (7, 21, 51). Biochemical studies and structural analyses of POU domain-DNA crystals have revealed that POU domain binding sites are composed of two variably spaced half sites, with the 5′ half site contacting the POU-specific domain and the 3′ half site contacting the POU homeodomain (18, 21).

An alternative mechanism of POU factor action is suggested from studies of Schwann cell-specific repression of gene transcription by SCIP/Tst-1/Oct-6 (27). Although this POU factor is capable of activating transcription of synthetic promoters in an octamer-dependent manner in HeLa and other cells (23, 44, 51), it is a strong and specific repressor of various promoters in Schwann cells (27). In transient-cotransfection experiments, SCIP was shown to repress the myelin P_0, myelin basic protein, and low-affinity nerve growth factor receptor promoters but not strong viral promoters. Bacterially expressed SCIP binds with high affinity to several A+T-rich sequence motifs positioned upstream of the P_0 gene (12, 25). However, mutation or deletion of these sites has little or no effect on the ability of SCIP to repress (25). Thus, SCIP is postulated to repress transcription via specific interactions with Schwann cell-specific transcription factor partners that act directly on promoters (25). Similarly, promoters of the K5 and K14 keratin genes are specifically repressed by cotransfection of an Oct-6 effector into cultured keratinocytes, but Oct-6 binding to these promoters cannot be detected (6).

Most POU domain factors studied to date possess transactivation domains that are positioned on the amino-terminal side of the POU domain (1, 8, 16, 22, 23, 26, 29, 45). In the case Oct-1 and Oct-2, however, activation domains are present on both the amino- and carboxy-terminal sides of the POU domain, and these activation domains can discriminate among different promoters, probably as a result of specific protein-protein interactions with components of different basal transcription complexes (8, 46). Thus, in general, POU factors can

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be viewed as modular transcription factors in which a separable activation domain is recruited to the promoter through the interaction of the POU domain with DNA.

We have investigated a possible interaction of POU factors with neurotransmitter receptor gene transcription because of the partial overlapping patterns of POU gene expression and neuronal nicotinic acetylcholine receptor (nAchR) gene expression in the brain (56). The rat adrenal chromaffin tumor line PC12 is being used as a model system to investigate POU function in a neural context because this line expresses several markers of neuronal phenotype such as several neuronal nAchR genes and responds to nerve growth factor by differentiating into sympathetic neuron-like cells (9). Recently, we found that SCIP strongly activates the nAchR α3 gene promoter in these cells but not in several nonneural cell lines (56). Footprint analysis revealed five SCIP binding sites positioned between −344 and −1470 in the nAchR α3 upstream region. However, elimination of these sites by either progressive 5′ deletion or point mutation of A+T-rich cores within binding sites failed to show a significant loss of α3 promoter transactivation by cotransfected SCIP (reference 56 and data not shown). Indeed, a reporter that contains the α3 core promoter but no detectable SCIP binding sites is strongly responsive to SCIP. One possibility is that in PC12 cells, the SCIP POU domain does not function simply to recruit its separable amino-terminal activation domain (23) to the DNA. Here, we investigate this hypothesis by comparing the transactivation properties of the SCIP polypeptide, GAL4-SCIP chimeras, and SCIP polypeptide truncations in PC12 cells. We find that the SCIP amino-terminal activation domain is dispensable for α3 activation and that the SCIP POU domain, by itself, is sufficient for transcriptional activation.

MATERIALS AND METHODS

Luciferase reporters. To prepare a reporter in which a SCIP binding site ($SCIP_{\alpha3}$) normally located 1.3 kb upstream of the α3 minimal promoter was moved immediately upstream of the promoter, α3-luciferase reporter −1607/+47-luc was cut with *Bst*1107I and *Pml*I to delete $SCIP_{\alpha3}$ minimal promoter interposing sequences and then religated. To prepare a reporter containing three copies of the $SCIP_{\alpha3}$ site, double-stranded oligonucleotides with the sequence 5′-CA GAA TTA ATG TAC GAA TTA ATG TAC GAA TTA ATG (upper) were annealed and substituted for the *Kpn*I-*Pml*I fragment of −1607/+47-luc. In the resulting reporters, the binding sites are approximately 200 bp upstream of the α3 transcription start site region. Underlined sequences comprise the $SCIP_{\alpha3}$ binding site.

To prepare a GAL4 binding-site reporter, one 17-bp GAL4 DNA element was cloned as a double-stranded oligonucleotide in pGL2 plasmid (Promega) digested with *Bam*HI and *Hin*dIII. The oligonucleotide sequences were 5′-GAT CCG GAG GAC TGT CCT CCG A (upper) and 5′-AGC TTC GGA GGA CAG TCC TCC G (lower). The resulting plasmid, pGL2-1XG, was cut with *Bsa*HI-*Bam*HI and *Bgl*II-*Bsa*HI, and the appropriate fragments were cross-ligated to produce plasmid pGL2-2XG, with two GAL4 sites. This plasmid underwent the same cloning step, which resulted in pGL2-4XG. A 161-bp *Kpn*I-*Hin*dII fragment that contains four GAL4 palindromes was cloned upstream of the *Pml*I site in the α3 promoter. The effector designated 4XG-238/+47-luc has four 17-bp GAL4 elements, each with a spacing of 16 bp followed by the α3 minimal promoter. A similar reporter (m4XG-238/+47-luc) bearing a mutated Sp1 binding site within the α3 core promoter was prepared from mutSp1-luc (55).

Effector plasmids. Effector constructs used in this study carry various portions of SCIP coding region between *Xba*I and *Kpn*I sites of the cytomegalovirus promoter-bearing pCGS vector (25, 45). Other SCIP deletion constructs were prepared from SCIP(3-451) by restriction endonuclease digestion using unique sites within the coding region and then religation. These constructs contain the following open reading frames: SCIP(3-451), masrssTTA..SVQ; SCIP(145-451), masrsldPGA..SVQ; SCIP(234-451), masrsgAGG..SVQ; SCIP(3-408), masrssTTA..PPMavpd; and SCIP(234-408), masrsgAGG..PPMavpd. Lowercase letters designate cloning derived residues. Effector SCIP(247-399) (open reading frame masrsEDA..MTPavpd) was prepared by PCR using *Pfu* polymerase (Stratagene) and *Xba*I-*Kpn*I subcloning into pCGS. The oligonucleotides used for PCR were 5′-CAC TCT AGA GAG GAT GCT CCC AGC TCC (upper) and 5′-CGC GGG TAC CGC GGG GGT CAT GCG CT (lower) (the *Xba*I and *Kpn*I sites are underlined).

To prepare a GAL4-SCIP chimera, the DNA binding domain of GAL4 (amino acids 2 to 148) was amplified with *Pfu* polymerase by using the following oligonucleotides: 5′-GCG CAC CTG CAC CCA GGC AAG CTA CTG TCT TCT ATC GAA C (upper) and 5′-GTC GTC CAT GGG CGA TAC AGT CAA CTG TC (lower) (the *Bsp*MI and *Nco*I sites are underlined). The 468-bp PCR fragment was cloned into *Bsp*MI-*Nco*I-digested pCGS-SCIP. The open reading frame in this construct, SCIP(3-233)GAL4(2-148)SCIP(407-451), is masrssTTA ..HPGkll..vspMD..SVQ (the GAL4 sequences are underlined, and the SCIP sequences are in capital letters). The amino-terminal deletion of the chimeric factor, GAL4(2-148)SCIP(407-451), and the GAL4 DNA-binding domain alone, GAL4(2-148), were prepared similarly to the SCIP(234-451) and SCIP(3-408) effectors and code for polypeptides masrsgkll..vspMD..SVQ and masrsg kll..vspMavpd. In the GAL4-VP16 effector (kindly provided by Hsing-Jien Kung), the simian virus 40 promoter drives the expression of a polypeptide fusion between the GAL4 DNA binding domain and the acidic activation domain of VP16.

All effector constructs were checked by diagnostic restriction digests and tested for quantitative expression of functional protein by electrophoretic mobility shift assay with the appropriate oligonucleotide probes.

Cell culture and transfections. Cells were grown as described previously (56). Transient transfections were performed by electroporation, using a Bio-Rad Gene Pulser with the capacitance extender and 0.4-cm cuvettes as described previously (56). Cells were transfected with 10 μg of reporter, the indicated quantity of POU effectors and pSP64 as the carrier. A total of 25 μg of DNA was used for each electroporation. Expression plasmid pRSV-βgal (5 μg) was used in some electroporations to control for transfection efficiency. Cells were electroporated at 300 V and 960 μF. Following each discharge, the DNA-cell suspension was transferred immediately to 60-mm-diameter dishes containing 5 ml of cell culture medium, which was held at room temperature. Cultures were then incubated under standard conditions for approximately 48 h. Cell extracts were prepared by using luciferase cell lysis reagent obtained from Promega. Luciferase assays were performed as previously described (56). Protein content of extracts was determined with Bio-Rad DC protein assay reagents.

Electrophoretic mobility shift assay. Nuclear extracts of transfected PC12 cells were prepared as described by Schreiber et al. (39). Nuclear pellets were resuspended in 50 μl of buffer C (39). Bacterially expressed SCIP protein was obtained by using the QIAexpress system as described previously (56). Double-stranded oligonucleotides used in competition-binding assays with bacterially expressed SCIP were (i) rat P_0 gene upstream sequence, 5′-GTA GAA AGA ACT GAA TTA CCA TTC TAA TAC GAG, which contains a high-affinity SCIP binding site (12), (ii) SCIP binding site, designated $SCIP_{\alpha3}$, 5′-GTT TTG TTT TTA GAA TTA ATG TAC AAT AAA G, present in the α3 upstream region; and (iii) a substituted version (substitutions in lowercase), 5′-GTT TTG TTT TTA Ggg ccg ATG TAC AAT AAA G, of the $SCIP_{\alpha3}$ oligonucleotide. Underlined sequences constitute A+T-rich cores of SCIP binding sites. An octamer containing double-stranded oligonucleotide 5′-GAT CAG TAC TAA TTA GCA TTA TAA AG (upper) was used for mobility shift assays (32) with extracts prepared from transfected PC12 cells.

Binding reactions with bacterially expressed SCIP (20 μl) were performed in 0.5× Tris-glycine buffer (25 mM Tris [pH 8.5], 190 mM glycine, 1 mM EDTA), 10% glycerol, 1.5 μg of poly(dI-dC), 0.05 pmol of probe, and indicated quantities of partially purified SCIP or protein from an empty vector (pQE10)-transformed extract. Binding reactions with octamer probe and PC12 extracts were performed with 4 to 6 μl of extract, 2 μg of poly(dI-dC), 1 μl of probe (10^5 cpm/μl), and buffer C to bring the final volume to 15 μl. All binding mixtures were incubated for 30 min at room temperature and loaded on either 4 or 6% polyacrylamide gels run in 0.5× Tris-glycine buffer (cross-link, 30:1).

RNase protection analysis. Because of low transient-transfection efficiencies in PC12 cells, electroporation conditions were first optimized to increase the number of transcripts produced from supercoiled DNA templates. We found that two consecutive discharges at 960 μF and 300 V for each transfection increased luciferase activities 20- to 40-fold per microgram of protein. For each electroporation, 10 μg of −238/+47-luc reporter and 1 μg of either SCIP effector or empty pCGS vector were used and balanced to 25 μg with pSP64. Transfected cells from six similar electroporations were combined in a 150-mm-diameter tissue culture dish and incubated for 36 h. Total RNA was isolated by using RNeasy kit (Qiagen, Inc.), with typical yields of 40 μg per dish. RNA preparations were then DNase I treated in the presence of RNase inhibitor and stored at −80°C until needed.

The probe template was made by cloning a 281-bp *Xba*I-*Nar*I fragment from the −238/+47-luc reporter into pGEM-3Z cut with *Xba*I and *Sma*I. The antisense probe (302 nucleotides) was transcribed by T7 RNA polymerase (Boehringer Mannheim) for 2.5 h at 32°C in buffer supplied with the enzyme. The transcription reaction mixture contained 0.5 μg of gel-purified DNA template cut with *Xba*I and 50 μCi of [α-^{32}P]UTP (800 Ci/mmol). The probe was ethanol precipitated, purified on a 4% denaturing polyacrylamide gel, and used within a week of synthesis.

Protection assays were performed with an RPA II kit (Ambion) essentially as recommended by the manufacturer. Thirty-five-microgram RNA samples were balanced to 50 μg with yeast tRNA and precipitated with 20,000 cpm of probe. Following hybridization at 43°C, the samples were digested with a mixture of 2 μg RNase A and 40 U of RNase T_1 for 45 min at room temperature. After

ethanol precipitation, the samples were run on a 5% denaturing polyacrylamide gel. The gel was dried and exposed to an X-ray film for 7 days or analyzed with a PhosphorImager analyzer (Molecular Dynamics).

RESULTS

The GAL4 DNA binding domain cannot functionally replace the SCIP POU domain. If the SCIP POU domain functions merely to recruit its separable amino-terminal activation domain (23) to the DNA, then a heterologous DNA binding domain should be capable of functionally replacing the SCIP POU domain. We investigated this possibility by substituting the SCIP POU domain with the GAL4 DNA binding domain and then comparing the characteristics of α3 promoter activation by wild-type SCIP and the GAL4-SCIP chimera.

To facilitate this comparison, we determined the SCIP responses of α3 minimal promoter constructs bearing either one or more copies of a SCIP binding site, $SCIP_{\alpha3}$, located about 1.3 kb upstream of the α3 promoter (56). This site was first characterized by mobility shift assay to demonstrate its ability to bind specifically to bacterially expressed SCIP. As shown in Fig. 1A, the binding of SCIP to a high-affinity binding site present in the myelin P_0 promoter (12) was competed for by excess unlabeled $SCIP_{\alpha3}$ (lanes 4 to 7) but not by an oligonucleotide in which the A+T-rich core of $SCIP_{\alpha3}$ was destroyed (lanes 8 to 10). When equivalent amounts of probe and SCIP were used, complex formation with $SCIP_{\alpha3}$ was greater than complex formation with the myelin P_0 SCIP binding site, suggesting that $SCIP_{\alpha3}$ has higher affinity for SCIP than does the P_0 site (Fig. 1B; compare lanes 2 and 3 with lanes 5 and 6). Scatchard analysis indicated that $SCIP_{\alpha3}$ has about twofold-greater affinity for SCIP than the P_0 site (data not shown).

When α3 minimal promoter constructs bearing either zero, one, or three copies of $SCIP_{\alpha3}$ were tested for SCIP activation in PC12 cotransfections assays, the activation dose responses for each were identical to one another (Fig. 1C). Since both footprint and mobility shift analyses with bacterially produced SCIP failed to show SCIP binding to the α3 minimal promoter (reference 56 and data not shown), these results show that SCIP activation of the α3 promoter does not depend on the presence of SCIP binding sites and that activation cannot be enhanced even when multiple copies of a high-affinity binding site are positioned immediately upstream of the α3 minimal promoter. It is also unlikely that SCIP responses are mediated by adventitious binding sites in the vector backbone or luciferase gene because (i) SCIP responses are specific to α3 and (ii) linearized reporters that were first gel purified away from vector backbone sequences remain strongly responsive to cotransfected SCIP (reference 56 and data not shown).

We then performed a similar analysis with the GAL4-SCIP fusion protein by using a reporter, 4XG-238/+47-luc, in which GAL4 binding sites were multimerized immediately upstream of the α3 minimal promoter. The results of cotransfection with the chimeric effector revealed that GAL4-SCIP was capable of activating the promoter, but with characteristics that were clearly distinct from those of wild-type SCIP. We found that GAL4-SCIP weakly activated at low effector masses. When the amount of transfected GAL4-SCIP effector was increased beyond 2 μg, however, severe squelching was observed (Fig. 2). This is in striking contrast to results for wild-type SCIP, in which case squelching is not observed over a comparable range of SCIP effector masses (Fig. 1C). A second significant difference between wild-type SCIP and the chimeric factor was that the weak but reproducible activation by GAL4-SCIP was binding site dependent, as no activation was seen in the absence of GAL4 binding sites (Fig. 3). This again contrasts with the data presented in Fig. 1C, which showed that the presence of SCIP binding sites positioned immediately upstream of the minimal promoter was without effect on the magnitude of transactivation. Activity of the chimeric factor was dependent on SCIP amino-terminal polypeptide sequences because no activity was observed with the GAL4 DNA binding domain alone (data not shown) or with a chimeric factor, GAL4-SCIPΔN, in which the GAL4 DNA binding domain was fused to the SCIP carboxy-terminal region (Fig. 3). The weak activation seen at low GAL4-SCIP effector masses would suggest that although the SCIP amino-terminal activation domain can act directly on the α3 promoter, it is a poor activation domain for α3 transcription in PC12 cells. This result is consistent with a previous report of a stringent cell type specificity of the SCIP amino-terminal activation domain (26). The different transactivation characteristics of wild-type SCIP and the GAL4-SCIP chimera as well as the weak activity of the SCIP amino-terminal activation domain emphasize the critical and novel role of the SCIP POU domain for activation. Since SCIP binding sites are not obligatory for SCIP activation, protein-protein interactions, mediated by the SCIP POU domain, appear to be essential for activation of α3 in PC12 cells.

A SCIP polypeptide truncation lacking the SCIP amino-terminal activation domain retains transcriptional activity. The preceding results led us to wonder which segments of the SCIP polypeptide are required for activation. We prepared effector constructs that lack different portions of the SCIP coding region and first tested each for octamer-binding activity in PC12 cells. All of the truncated effectors contained sequences encoding an intact SCIP POU domain but lacked various segments of the amino and carboxy termini. As shown in Fig. 4A, these truncations were of the expected relative sizes and produced comparable binding activities. Thus, there was no indication that deletion of different portions of the SCIP coding region significantly decreased binding affinity, protein stability, or nuclear localization. When tested in cotransfections, amino-terminal deletion of 144 residues [SCIP(145-451)] was without significant effect on promoter transactivation (Fig. 4B). Further deletion to residue 234 [SCIP(234-451)] resulted in a 50% decrease in activity, which is consistent with the presence of an amino-terminal activation domain (23, 25). The substantial activity of this effector, however, was unexpected because similar effectors, which lack amino-terminal activations domains, were reported to be unable to activate transcription via octamers in HeLa cells (23) or to repress the myelin P_0 promoter in Schwann cells (25, 26). Interestingly, the activity of an effector expressing little more than the POU domain [SCIP(234-408)] was comparable to that of the wild-type polypeptide. The greater activity of SCIP(234-408) than of SCIP(234-451) likely resulted from an increased level of expression of the SCIP(234-408) truncation and not from a transcriptional inhibitory domain in the carboxy terminus (data not shown). Our results demonstrate, therefore, that deletion of previously characterized SCIP amino-terminal activation domains (23, 25) does not destroy α3 activation in PC12 cells.

To show that activation of the α3 promoter by wild-type SCIP and the SCIP(234-408) truncation reflects an increase in the level of correctly initiated reporter transcripts, we performed RNase protection analysis with total RNA isolated from transiently transfected PC12 cells. Analysis of the α3 endogenous transcripts in PC12 cells revealed that transcription initiated at multiple sites positioned over an ~70-bp region (Fig. 5A, right panel) as was previously described (56). We then analyzed total RNA from PC12 cells that were transfected with −238/+47-luc in the presence or absence of SCIP effector plasmids. To specifically detect −238/+47-luc report-

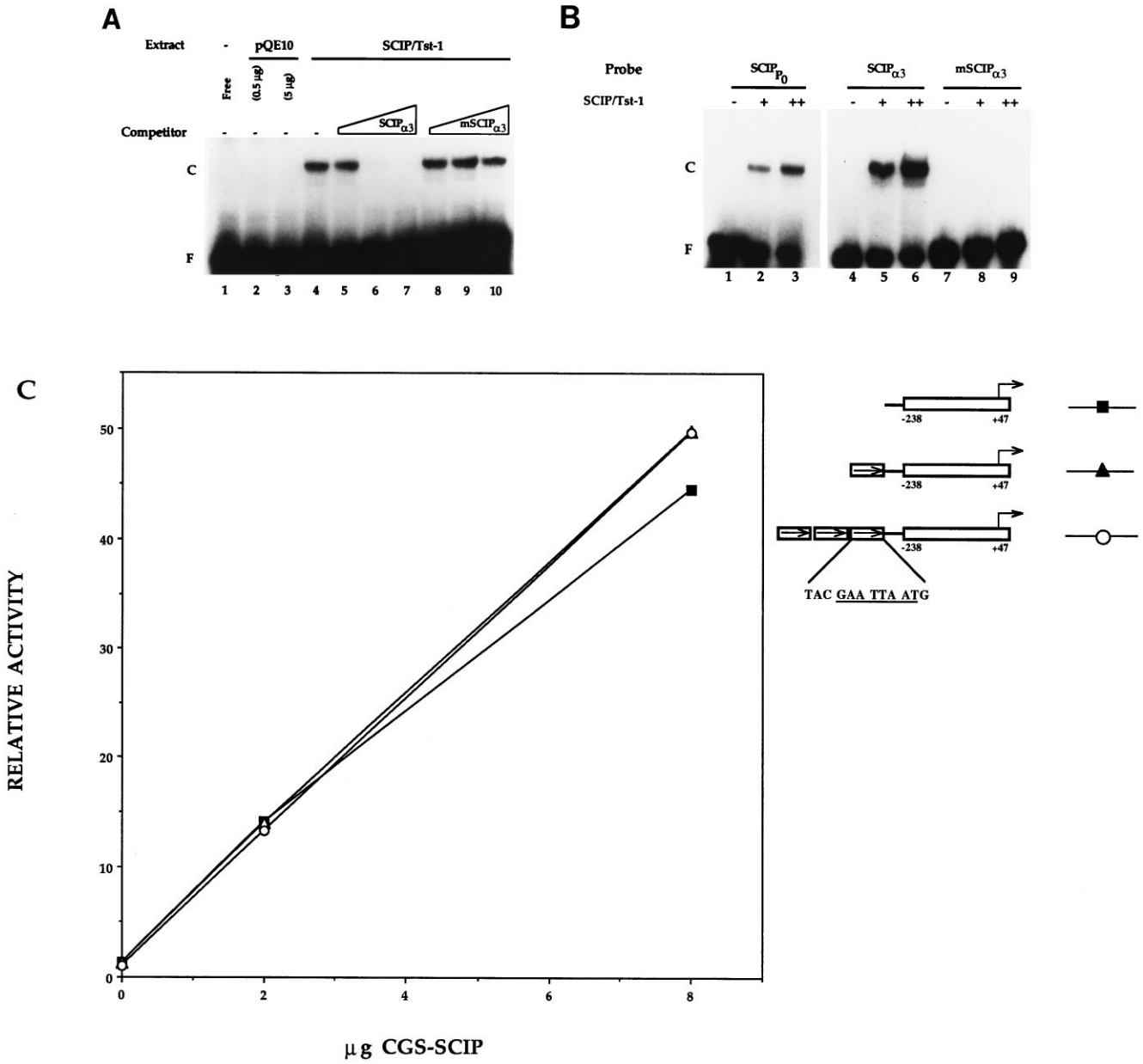


FIG. 1. SCIP responses of α3 minimal promoter constructs with and without high-affinity SCIP binding sites. (A) Competition binding assay was performed with 0.1 ng of a radiolabeled duplex oligonucleotide ($SCIP_{P0}$) bearing the highest-affinity SCIP binding site from the Schwann cell-specific P_0 upstream region and 20 ng of bacterially purified SCIP. Unlabeled competitors were an α3 nicotinic duplex oligonucleotide ($SCIP_{α3}$) containing a 6-of-8-nucleotide match to the P_0 SCIP binding site (lanes 5 to 7) or equivalent molar excesses of a mutated version of the nicotinic oligonucleotide ($mSCIP_{α3}$; lanes 8 to 10). For each competitor, molar excesses were 20-, 100-, and 500-fold. (B) Binding of SCIP to P_0 and α3 SCIP binding sites. Binding assays were performed with no protein (lanes 1, 4, and 7) or 20 ng (lanes 2, 5, and 8) or 60 ng (lanes 3, 6, and 9) of SCIP and double-stranded radiolabeled oligonucleotide $SCIP_{P0}$ (lanes 1 to 3), $SCIP_{α3}$ (lanes 4 to 6), or $mSCIP_{α3}$ (lanes 7 to 9). C, protein-DNA complex; F, free probe. (C) SCIP binding sites positioned immediately upstream of the α3 promoter do not modulate SCIP activation. PC12 cells were transfected with reporters containing either zero, one, or three copies of the SCIP binding site present in $SCIP_{α3}$ immediately upstream of the α3 core promoter (−238/+47). Dose responses were obtained for each of the reporters by cotransfection with the indicated masses of pCGS-SCIP. Protein corrected data are expressed relative to the basal activity of −238/+47, which was set to 1.

er-derived transcripts, we used a second probe that extended from the luciferase gene to a site upstream of the α3 transcription start site region (Fig. 5B). As shown in the left panel of Fig. 5A, cotransfection of effector plasmids expressing SCIP(3-451) or SCIP(234-408) resulted in a substantial increase (up to 20-fold) in the abundance of transcripts derived from the α3 start site region. The relative induced levels of specific reporter transcripts were similar for the two effectors and paralleled the relative basal levels of the different endogenous α3 transcripts (Fig. 5A, right panel). Thus, the activity of SCIP(234-408) is identical to that of wild-type SCIP, and both effectors stimulate transcription without an obvious preference for a specific subset of α3 start sites. In addition to activation of α3 transcripts, both effectors increased the abundance of a fully protected product, which may reflect a readthrough transcript produced from a SCIP-responsive cryptic promoter lying upstream of the

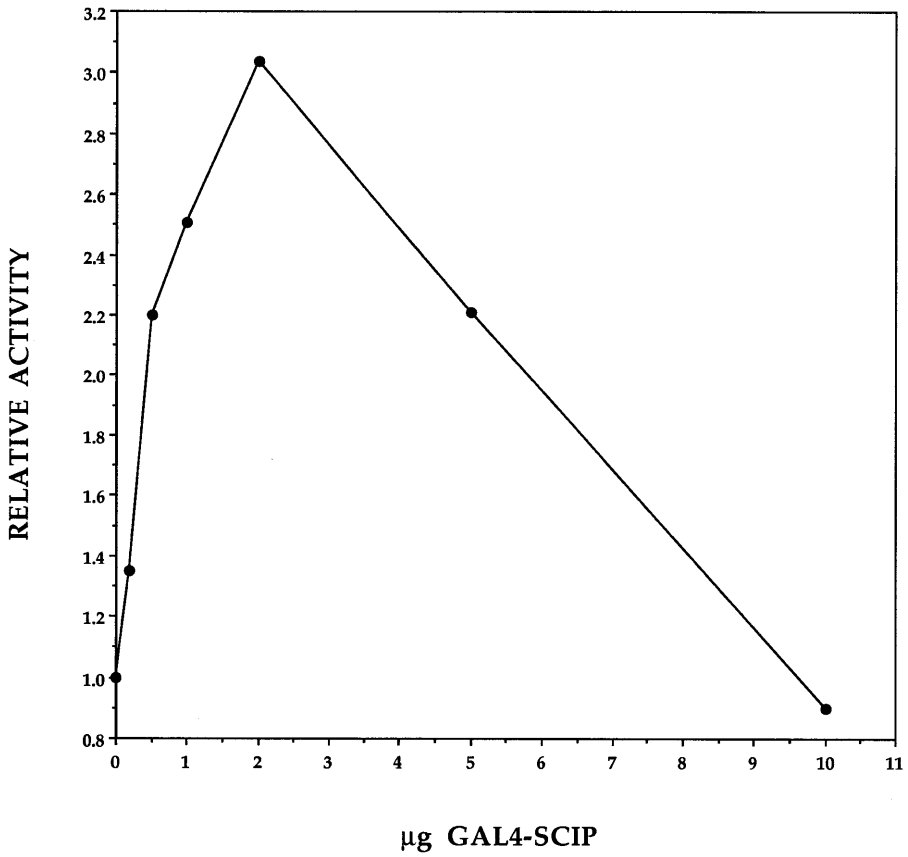


FIG. 2. GAL4-SCIP fusion protein activates and then squelches α3 transcription. Ten micrograms of an α3 minimal promoter construct, 4XG−238/+47-luc, bearing four 17-bp GAL4 DNA binding sites was cotransfected into PC12 cells with 0 to 10 μg of GAL4-SCIP and 5 μg of pRSV-βgal. The effector construct GAL4-SCIP contains yeast GAL4 DNA binding domain cloned in place of the POU domain between SCIP amino- and carboxy-terminal domains (see Materials and Methods). Relative luciferase activities were obtained and corrected for transfection efficiencies with β-galactosidase activities and then plotted against the effector concentration. The corrected activity at 0 μg of GAL4-SCIP effector was set to 1.

protected region. Nevertheless, these results show that activation of luciferase activity by wild-type SCIP and a truncated polypeptide comprising little more than the SCIP POU domain is correlated with a corresponding increase in the abundance of similar sets of correctly initiated α3 reporter transcripts.

The POU domain of SCIP is sufficient for activation. SCIP(234-408) encodes the SCIP POU domain and small amino- and carboxy-terminal flanking segments that contain short strings of glycine or alanine residues, which are conserved between rats and mice (12, 24, 27). To investigate whether these sequences were essential for the activity of the polypeptide, we prepared an additional effector [SCIP(247-399)] in which the SCIP coding sequences were truncated to express an intact SCIP POU domain but not flanking segments. Remarkably, we found that the activity of this polypeptide was nearly equivalent to that of wild-type SCIP, indicating that the short amino- and carboxyl-terminal flanking segments may contribute but are not necessary for activation (Fig. 6). Furthermore, these results demonstrate that the SCIP POU domain, by itself, is sufficient for activation in PC12 cells.

Coexpression of the SCIP POU domain with the GAL4-VP16 activator results in transcriptional synergism. The results presented thus far suggest that the DNA binding domain of SCIP may have the ability to function as a transcriptional activation domain in PC12 cells. An essential aspect of activation domains as regulators of gene expression is their capacity for transcriptional synergism (36). To determine whether coexpression of the SCIP POU domain and a second transcriptional activator results in synergistic activation, we cotransfected PC12 cells with the 4XG−238/+47-luc reporter, the SCIP(247-399) effector, and an effector expressing the GAL4 DNA binding domain fused to the VP16 acidic activation domain. When transfected in the absence of the SCIP effector, 10 μg of the GAL4-VP16 protein stimulated 4XG−238/+47-luc about 50-fold. When 2 μg of the SCIP(247-399) effector was transfected alone, it stimulated the reporter about sevenfold. However, when these effectors were cotransfected, reporter activity was stimulated in a multiplicative manner (Fig. 7A). Synergism was observed over a range of both SCIP(247-399) (1 to 2 μg)- and GAL4-VP16 (2 to 15 μg)-cotransfected effector masses (data not shown). These results show, therefore, that the DNA binding domain of a POU domain factor is not only sufficient for transcriptional activation but also likely to mediate synergism with an acidic activation domain.

Because transcriptional activation and synergism stimulated by the SCIP POU domain occur in the absence of SCIP binding sites, it is likely that the responses that we see result from protein-protein interactions that tether the SCIP POU domain to factors bound to the α3 minimal promoter. The structure of this promoter appears to be quite simple in that transcription initiates from multiple sites and is stimulated severalfold by an Sp1 motif located immediately upstream of the start site region. Other than the Sp1 motif, no *cis* elements that contribute

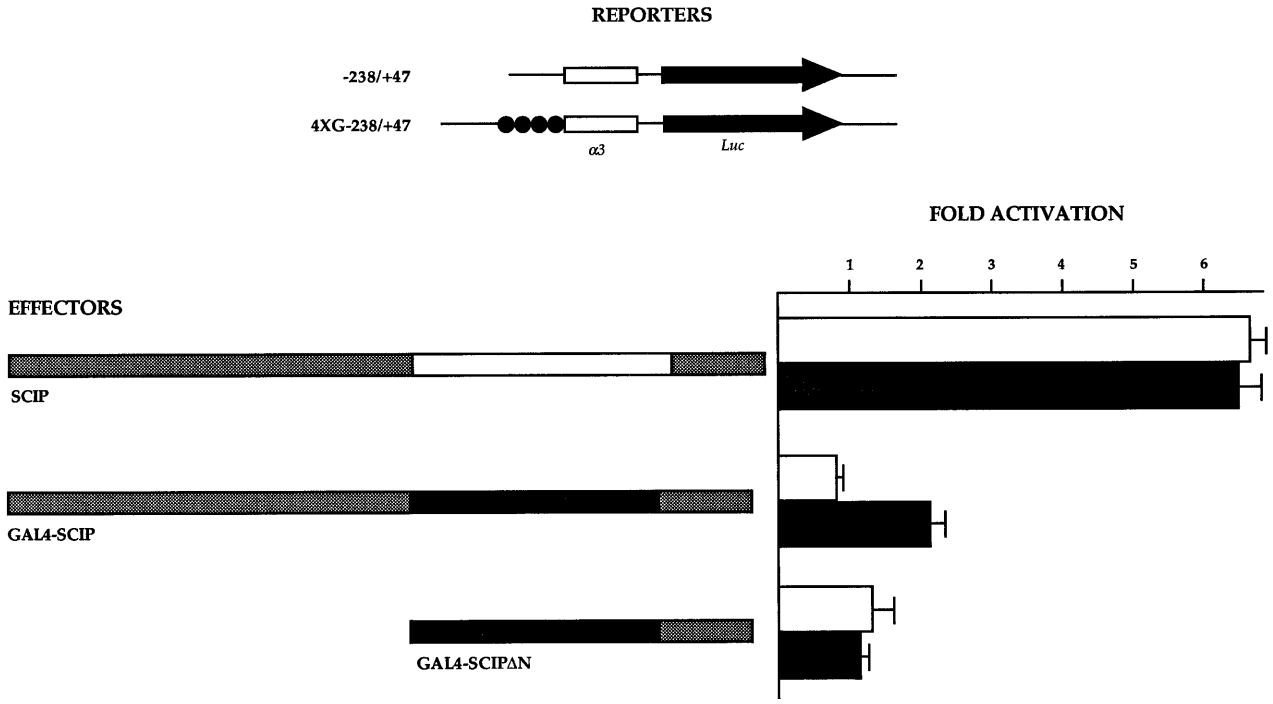


FIG. 3. GAL4 binding-site-dependent activation of the α3 promoter by GAL4-SCIP protein. The schematic at the top shows reporters used in this experiment. The −238/+47-luc reporter contains the α3 minimal promoter (open box), and the 4XG−238/+47-luc reporter contains four 17-bp GAL4 DNA binding sites (filled circles) multimerized immediately upstream of α3 sequences. At the lower left is a schematic of the effectors used in cotransfection assays. The effector GAL4-SCIPΔN was constructed by deletion of the SCIP amino terminus from GAL4-SCIP. The unfilled segment of the SCIP effector represents the POU domain; gray segments depict amino and carboxy termini of SCIP (amino acids 3 to 233 and 409 to 451, respectively), and black segments in GAL4-SCIP and GAL4-SCIPΔN represent the GAL4 DNA binding domain (amino acids 2 to 148). PC12 cells were cotransfected with 10 μg of either −238/+47-luc reporter (open bars) or 4XG−238/+47-luc reporter (filled bars) and 1 μg of the indicated effector. Fold activation is calculated as the ratio of activated level to the basal level for each reporter and is presented as a mean of n independent experiments ± standard error of the mean (n = 8 for SCIP and GAL4-SCIP effectors; n = 4 for GAL4-SCIPΔN).

significantly to α3 minimal promoter activity were identified (55). Thus the transcriptional activity of SCIP could arise through an interaction with factors bound to the Sp1 motif. On the other hand, the target of the SCIP POU domain is perhaps a component of the general transcription complex. In this case, the presence of the Sp1 motif should not be necessary for synergism. To begin to determine the mechanism through which SCIP activates α3 in PC12 cells, we investigated whether the Sp1 motif is required for synergistic activation. We prepared a reporter, m4XG−238/+47-luc, in which the Sp1 motif in 4XG−238/+47-luc reporter was mutated so that Sp1 factors are unable to bind (55). As shown previously for −238/+47-luc (55), mutation of the Sp1 motif in 4XG−238/+47-luc decreased α3 minimal promoter activity to 20% (data not shown). However, as shown in Fig. 7B, cotransfection of PC12 cells with SCIP(247-399) and the GAL4-VP16 effector resulted in synergistic activation of m4XG−238/+47-luc. Since the Sp1 motif is not obligatory for cooperative interactions with GAL4-VP16, these results suggest that SCIP interacts with components of the general transcription complex to activate synergistically.

DISCUSSION

In this study, we have analyzed the polypeptide domains of SCIP/Tst-1/Oct-6 involved in neuronal nAchR α3 promoter activation. The main findings presented here are that (i) substitution of the SCIP POU domain with the GAL4 DNA binding domain creates a novel effector that can act directly on α3 but with transactivation characteristics that are clearly distinct from those of wild-type SCIP, (ii) the SCIP amino-terminal activation domain is dispensable for activation, (iii) the SCIP POU domain by itself is transcriptionally active, and (iv) coexpression of the SCIP POU domain and the GAL4-VP16 activator results in synergistic activation of α3. Thus, our results demonstrate that in PC12 cells, the SCIP POU domain does not function merely to recruit its separable amino-terminal activation domain to the DNA.

Activation of α3 by wild-type SCIP and by GAL4-SCIP. Comparative analysis of wild-type SCIP and a chimeric GAL4-SCIP factor provides support for a novel role of the SCIP POU domain in the context of α3 promoter activation. We found that when the SCIP POU domain was replaced by the yeast GAL4 DNA binding domain, activation characteristics of α3 were dramatically different. At low concentrations the chimeric factor activated in a binding-site-dependent manner. This result differs from the responses obtained with wild-type SCIP, in which activation could not be modulated even when one or more well-characterized SCIP binding sites were positioned immediately upstream of −238/+47-luc. Thus, forced recruitment of SCIP close to the promoter via a specific interaction with DNA was without effect. These results contrast with SCIP repression of the myelin P_0 gene, in which case repression does not depend on the presence of SCIP binding sites in their native positions, but these sites can mediate repression if moved much closer to the P_0 transcription start site (25). Another major difference was that squelching was observed with GAL4-SCIP over a range of concentrations of effectors that do not result in squelching by wild-type SCIP. Furthermore, the maximum level of activation by the GAL4-SCIP chimera was

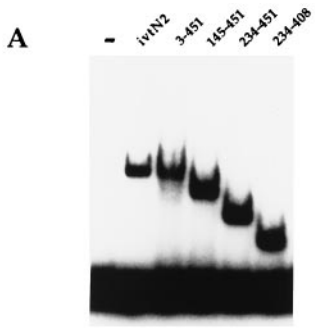
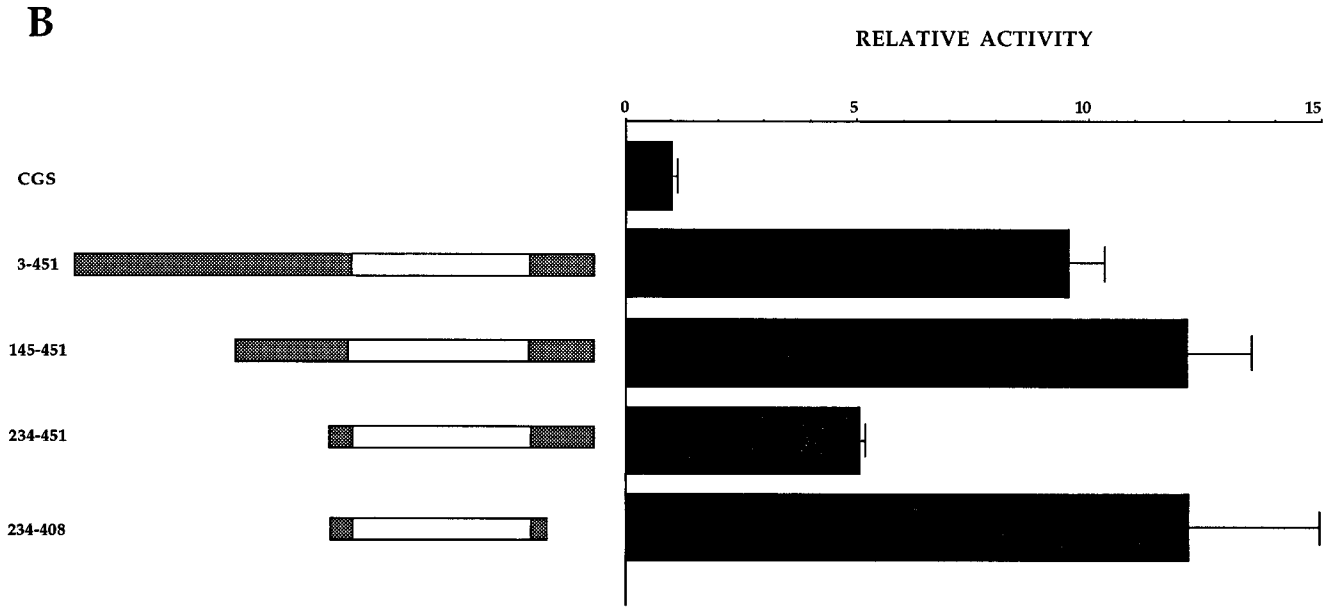


FIG. 4. SCIP can activate α3 in the absence of its amino-terminal activation domain. (A) Octamer binding activity of SCIP polypeptide truncations expressed in PC12 cells. Effector designations in numbers refer to SCIP amino acid residues. For each effector, 10 μg plasmid was electroporated, and nuclear extracts were prepared 2 days later. Equivalent amounts of protein from each extract was incubated with an end-labeled octamer probe. Shown are results for free probe (lane 1 from left), in vitro-translated SCIP (ivtN2; lane 2), and extracts prepared from cells transfected with the indicated pCGS-SCIP truncations (lanes 3 to 6). (B) Activity of SCIP polypeptide truncations. Unfilled portions of each truncation schematic represent the POU domain. Cotransfections were performed with 10 μg of −238/+47-luc and 2 μg of the indicated effector. Relative light units were corrected for protein content of cell lysates. The results represent averages of data collected from several different transfection experiments and are expressed relative to the basal activity of −238/+47-luc, which was set to 1.



substantially lower than that obtained by wild-type SCIP. Thus, the GAL4 DNA binding domain is not functionally equivalent to the SCIP POU domain in these assays, and therefore the latter appears to mediate protein-protein interactions that may or may not require direct interaction with DNA. It is possible that SCIP transactivation of the α3 promoter requires interactions with low-affinity sequences that were not detected by footprint analysis (56). However, a number of lines of evidence argue against this possibility. First, the −238 to +47 sequence is G+C rich, and thus consensus POU binding sites are not apparent. Second, progressive 5′ deletion analysis of the minimal responsive segment down to basal promoter elements did not reveal a region essential for activation (data not shown). Third, SCIP binding to α3 minimal promoter sequences could not be detected by mobility shift assay (data not shown). Fourth, we showed previously (56) that a SCIP effector carrying mutations in the recognition helix of the POU homeodomain, which destroys its ability to bind DNA, is still able to partially activate the α3 promoter. Our results, therefore, do not support a mechanism in which SCIP activates α3 via direct interaction with *cis*-acting sequences lying outside the core promoter region. It remains possible, however, that SCIP makes contact with sequences in the core promoter region as a result of interaction with a basal transcription factor. Similar conclusions were drawn previously for repression of the myelin P_0 promoter by SCIP in Schwann cells (25).

The SCIP POU domain is sufficient for activation. Using both luciferase assays and RNase protection analysis, we determined the activities of several SCIP polypeptide truncations to investigate which regions contribute to activation in PC12 cells and whether these are similar to those reported for SCIP (Oct-6) in HeLa (23) and Schwann (25) cells. Our results revealed some similarities to those described for HeLa and Schwann cells, but the most notable feature of our analysis was the dramatic differences observed in PC12 cells. Consistent with studies of SCIP (Oct-6) in HeLa (23) and Schwann (25) cells, SCIP polypeptide sequences amino terminal to the POU domain contributed to α3 promoter modulation in PC12 cells in the context of either the SCIP POU domain or the GAL4 DNA binding domain. The amino-terminal region contributing to activation mapped to a segment between residues 145 and 234, since deletion up to residue 145 was without effect but deletion to 234 resulted in a 50% loss of activity. This aspect of SCIP activity, however, is clearly different from that in Schwann cells (25, 26), in which deletion to residue 145 abolished entirely the activity of the SCIP polypeptide, and in HeLa cells, in which deletion to residue 157 abolished activity of Oct-6 (23). In fact, in Schwann and HeLa cells, no truncation showed significant activity unless particular polypeptide segments amino terminal to the POU domain were included along with an intact POU domain. Moreover, Weinstein et al. (53) have recently shown that in Schwann cell cotransfections,

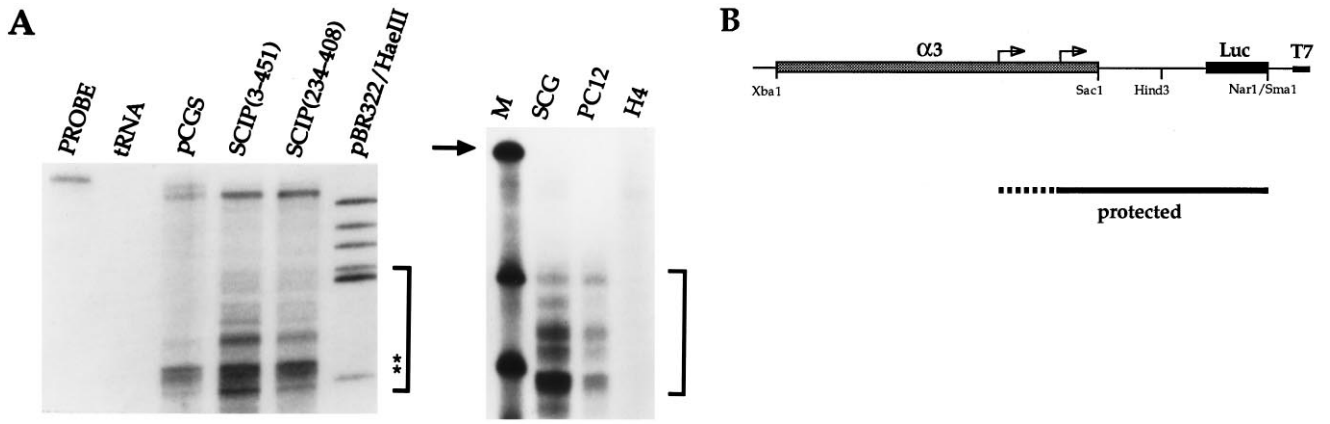


FIG. 5. RNase protection analysis of α3 promoter activation by SCIP effectors. (A) Left, analysis of chimeric α3-luciferase reporter transcripts expressed in PC12 cells after transfection with −238/+47-luc reporter and either the indicated SCIP effector plasmid or the pCGS empty effector plasmid. The bracket on the right designates the range of protected transcripts initiated at the α3 promoter start site region. The asterisks mark nonspecific protection products apparent in any PC12 cell RNA sample under the conditions used. tRNA, yeast tRNA hybridization; pBR322/HaeIII, DNA size markers. Exposure time was 7 days. Right, analysis of endogenous α3 transcripts presented as reference for reporter-derived transcripts shown in the left panel. Total RNA was isolated from rat superior cervical ganglia (SCG), PC12 cells, or H4 hepatoma cells. The RNA size markers in lane M were prepared by in vitro transcription with T7 RNA polymerase from appropriate linear DNA templates. The sizes of transcripts in nucleotides are 390, 256, and 207. The arrow on the left indicates the transcript that was used as the RNase protection probe. The bracket on the right designates the range of protected α3 transcripts, which reflects different transcription start sites (56). (B) Schematic of the chimeric α3-luciferase RNase protection probe. For preparation of the template plasmid, see Materials and Methods. Arrows designate boundaries of the α3 transcription start site region. The dotted portion of the "protected" line designates the range of α3 promoter-specific protected fragments.

a SCIP truncation identical to SCIP(145-451) and therefore missing the first 144 amino acids of the SCIP polypeptide acts not as an activator but as a dominant negative repressor of wild-type SCIP. Thus, the clear difference in activity of this effector in Schwann cells, HeLa cells, and PC12 cells demonstrates the cell-type-specific activity of SCIP subdomains and further supports the idea that SCIP activity may not be limited to a single mechanism.

Even more surprising was the activity in PC12 cells of SCIP polypeptides truncated from both the amino- and carboxy-terminal ends but which leave the POU domain intact. The activity of the SCIP polypeptide truncated to residue 234 on the amino-terminal end and to residue 408 on the carboxy-terminal end was similar to that of the wild-type polypeptide in PC12 cells. The increased activity observed upon deletion of the carboxy terminus appears to result in an increased level of protein (data not shown) and not from the loss of a transcriptional inhibitory domain within the SCIP carboxy terminus, which is reminiscent of Oct-2A carboxy-terminal truncations (29). RNase protection mapping indicated that both SCIP and a polypeptide composed of little more than the SCIP POU domain activated the same set of transcripts throughout the entire α3 multi-start site region, suggesting a common mechanism of action. Further deletion of amino acid residues up to both the amino- and carboxy-terminal boundaries of the POU domain resulted in only a small loss of activity. These findings suggest that the DNA binding domain of SCIP may function, alternatively, as a transcriptional activation domain in particular cell types.

Significantly, we also found that coexpression of the SCIP POU domain with the GAL4-VP16 acidic activator resulted in synergistic activation. Tst-1 was previously shown to activate transcription synergistically with papovavirus large tumor antigen (33). However, synergism with large tumor antigen is believed to require direct protein-protein interactions between the two activators and was dependent on the presence of Tst-1 binding sites (33). In contrast, synergism between the POU domain of SCIP and VP16 activation domain occurred in the absence of SCIP binding sites. Synergism also occurred in the absence of the homeodomain recognition subdomain of VP16, which functions to recruit the VP16 acidic activation domain to promoters through a protein-protein interaction with the Oct-1 POU homeodomain (40). The absence of this VP16 subdomain in the GAL4-VP16 fusion protein suggests that a direct protein-protein interaction between the SCIP POU domain and the GAL4-VP16 activator is not likely to occur during synergistic activation of α3. Thus, the SCIP POU domain may bear an alternative activation domain that can synergize with a different activation domain at the level of the basal transcription complex (36).

Although the major activation domains of POU factors are separable from their DNA binding domains, some studies have provided evidence that isolated POU domains can play a direct role in activation (29, 30, 46). However, some characteristics of activation by these other isolated POU domains are distinct from those reported here for SCIP. One difference is that their activities were significantly less than those of their wild-type counterparts. A second difference is that activation was dependent on the presence of POU binding sites. For the Oct-2A POU domain, activation was observed only when octamer-binding sites were positioned very close to a TATA box (29). Interestingly, a chimeric Oct-1/Pit-1 POU domain could activate a readthrough transcript, which is likely to be derived from a cryptic mRNA-type promoter, but could not activate correctly initiated transcripts from a downstream U2 small nuclear RNA promoter (4, 13, 46). Perhaps the cryptic mRNA-type promoter and the α3 promoter share characteristics that confer responsiveness to isolated POU domains.

Mechanism of SCIP activation in PC12 cells. How might the SCIP POU domain activate transcription in the absence of SCIP binding sites? Because we have not been able to demonstrate a functionally relevant site of interaction between SCIP and the α3 promoter, our data lead us to suggest that protein-protein interactions are essential. One possibility for activation alluded to above is that SCIP may modulate transcription via interactions with a second transcription factor that binds in a sequence-specific manner to a site in the α3 core region. The only apparent candidate for this type of interaction

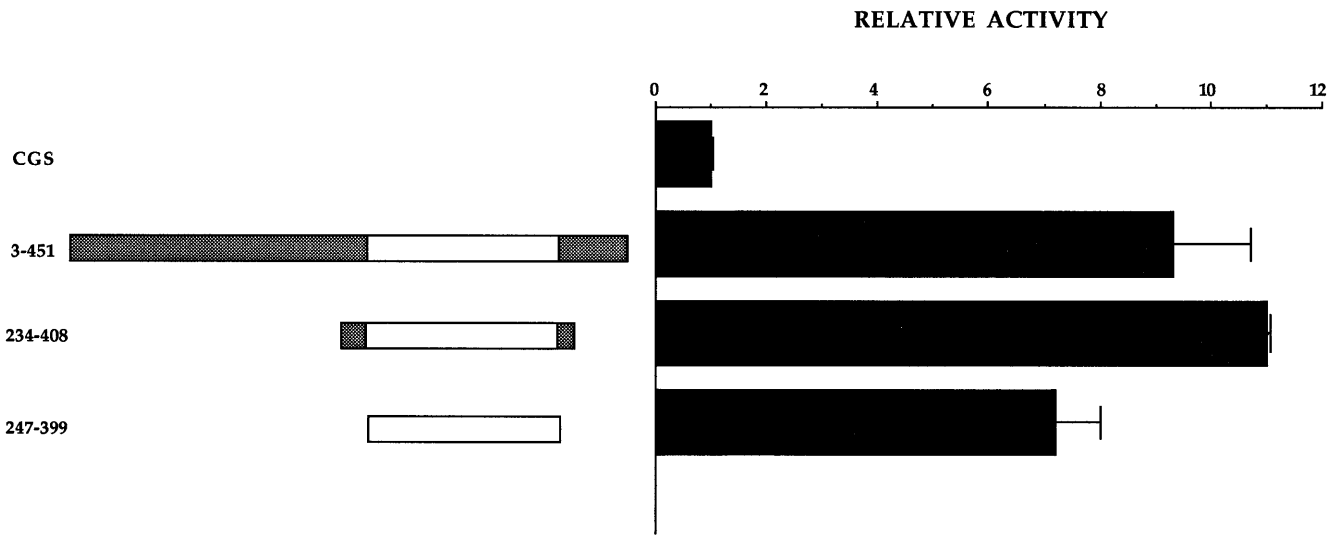


FIG. 6. The SCIP POU domain by itself is sufficient for promoter activation in PC12 cells. Effector designations in numbers refer to SCIP amino acid residues. The unfilled portion of each truncation schematic represents the POU domain. Cotransfections were performed with 10 μg of −238/+47-luc and 2 μg of the indicated effector. The results were obtained and are expressed as described for Fig. 4.

is Sp1, since the core promoter defined in PC12 cells is composed of a multi-start site region and a single upstream Sp1 site (55). However, because mutation of the Sp1 motif does not abolish synergistic activation upon coexpression with GAL4-VP16, an obligatory interaction with factors bound to this motif is not likely. Our results, therefore, point to protein-protein interactions with components of the basal transcription complex. SCIP may modulate a rate-limiting activity of the general transcription complex, or perhaps it titrates out a repressor bound to basal factors. Given the numerous examples of protein-protein interactions among different POU domains (reviewed in reference 48) and the observation that the SCIP POU domain, by itself, is sufficient for activation in PC12 cells, either of these two models seems plausible.

It is notable that several other sequence-specific transcription factors are thought to be capable of modulating transcription via interactions with the basal components. These include the zinc finger protein YY1 (34) and the myogenic factor MyoD (5). We have noted (56) the remarkable parallel of our results to the action of SCIP as a repressor in Schwann cells, in which SCIP binding is not required for SCIP to repress various promoters (25). Similarly, the homeodomain protein, Msx-1,

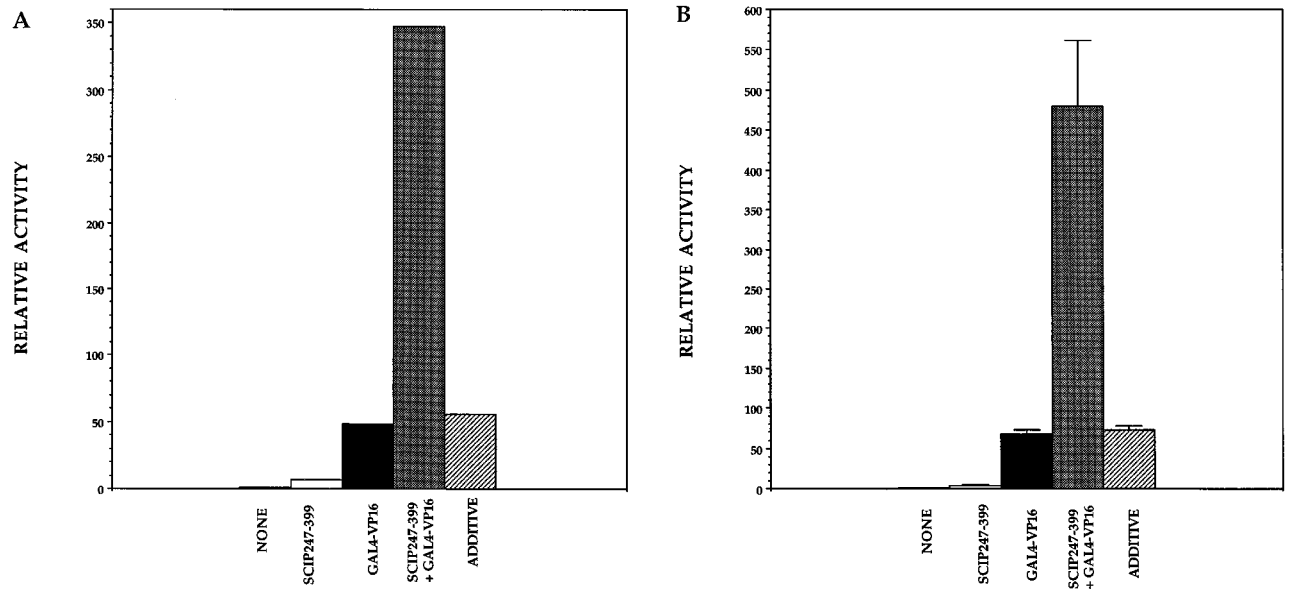


FIG. 7. Synergistic activation of α3 by coexpression of the SCIP POU domain and the GAL4-VP16 activator. (A) PC12 cells were transfected with 10 μg of the 4XG−238/+47-luc α3 reporter bearing multimerized GAL4 binding sites in the absence or presence of the indicated effectors. Effector quantities used were 10 μg for the GAL4-VP16 plasmid and 2 μg for SCIP(247-399). (B) Synergistic activation does not depend on the Sp1 motif upstream of the α3 transcription start site region. PC12 cells were transfected with a reporter, m4XG−238/+47-luc, bearing a mutation in the Sp1 motif of the α3 minimal promoter. Cotransfections with indicated effectors were performed as described above. Relative activity is presented relative to the basal activity of the reporters, which was set to 1. Bar marked "additive" represents the sum of individual reporter activities in the presence of either SCIP(247-399) or GAL4-VP16 effectors.

which can bind DNA in a sequence-specific manner, has also been shown to regulate transcription in the absence of homeodomain DNA binding sites (3). Similar to SCIP in Schwann cells, Msx-1 is also a potent repressor of transcription, which is proposed to result from protein-protein interactions with the general transcription complex (3). The mechanism of Msx-1 repression provides additional support for the view that particular activities of homeodomain proteins are not dependent on sequence-specific DNA binding activity (3). Another example of a homeodomain protein regulating transcription in the absence of homeodomain DNA binding sites is the pHOX1 protein, which activates by enhancing the DNA binding activity of serum response factor via protein-protein interactions (10). As far as we know, the ability of SCIP to activate in the absence of SCIP binding sites is the only example of an intact POU domain factor with this property, and thus it may constitute a novel example of the importance of protein-protein interactions for some aspects of homeodomain function (11). Although we have not formally excluded an indirect action of SCIP in which SCIP regulates another gene which in turn produces a protein that directly regulates α3, we believe it is less likely than a direct effect, given the ability of the GAL4-SCIP protein to act directly on α3 when recruited to the α3 promoter via multimerized GAL4 binding sites. An indirect effect seems less likely also because it would involve specific regulation of an intermediary factor by the POU domain of SCIP that is capable of synergism with the GAL4-VP16 protein. In addition, the partial activation seen with a SCIP mutant (56) that is unable to bind DNA argues against binding-site-dependent activation of an intermediate gene. Nevertheless, because an intact SCIP POU domain, by itself, is sufficient for activation and synergism, an indirect effect on α3 would still require a novel mechanism of action that ultimately results in modulation of α3 promoter activity.

In conclusion, our results identify a novel activity for the SCIP polypeptide in PC12 cells, in which the POU domain of SCIP, by itself, contains all of the protein interfaces required for transcriptional activation. This activity may reflect an alternative mode of transcriptional activation by SCIP in specific cellular contexts.

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