Activity of the Distal Positive Element of the Peripherin Gene Is Dependent on Proteins Binding to an Ets-like Recognition Site and a Novel Inverted Repeat Site*

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The peripherin gene, encoding a neuron-specific intermediate filament protein, is transcriptionally induced when PC12 cells begin to terminally differentiate into neurons in response to nerve growth factor. Previously we identified two regulatory sequences of the peripherin gene: a proximal negative element (centered at −173), which prevents peripherin expression in undifferentiated PC12 cells, and a distal positive region (−2660 to −2308) necessary for full induction of peripherin in differentiated PC12 cells (Thompson, M., Lee, E. L., Lawe, D., Gizang-Ginsberg, E., and Ziff, E. (1992) Mol. Cell. Biol. 12, 2501-2513). Here we define a distal positive element (DPE, −2445 to −2337) within the distal positive region. Methylation interference footprinting of the DPE identified DNA-protein contact points at a novel inverted repeat sequence (AACCACCTGGTT) and an Ets-like recognition sequence (CAGGAG). Functional analysis using site-directed mutagenesis demonstrates that both sites are necessary for the activity of the DPE. In addition, ternary complex formation at the DPE is dependent on both sites. Antibody competition assays confirm that an Ets family member participates in the DNA-protein complex. We have indirect evidence that the inverted repeat binding protein and the Ets-related protein interact directly with each other. Finally, we demonstrate that the DPE is constitutively active and that neuron-specific regulation of peripherin expression may be due to interaction with distal and proximal negative regulatory elements.

The neuron-specific intermediate filament proteins are a major component of the neuronal cytoskeleton. They include NF-L, NF-M, and NF-H, as well as peripherin, α-internexin, and nestin (reviewed in Refs. 2-4). They are expressed in different regions of the nervous system: NF-L, NF-M, and NF-H are found in all neurons, whereas α-internexin is expressed in the central nervous system (5), nestin in neuroepithelial stem cells (6), and peripherin in the peripheral nervous system and a subset of central nervous system neurons (7-10). During development, the expression of these genes is closely associated with the terminally differentiated neuronal phenotype (5, 6, 8, 11, 12). Identification of regulatory proteins that control the expression of these neuronal structural genes may lead to understanding of the cellular mechanism underlying neuronal differentiation.

We have studied the regulation of the peripherin gene, which encodes a neuron-specific type III intermediate filament protein (9, 10, 13, 14). The peripherin gene is a late response gene expressed approximately 12 h after initiation of nerve growth factor (NGF)† treatment of PC12 cells, corresponding to the time when PC12 cells begin to exhibit a neuronal phenotype (9, 15-17). In vivo, peripherin expression is limited to sympathetic, parasympathetic, and sensory ganglia of the peripheral nervous system as well as a small subset of neurons in the central nervous system (7, 9, 10). Peripherin is first expressed at day 11.5 of rat embryogenesis in the newly formed sympathetic ganglia (8). Belecky-Adams et al. (18) demonstrated that a transgene containing 5.8 kilobases of peripherin 5′-flanking sequence achieved correct temporal and nervous system-specific expression in transgenic mice, although intragenic sequences contribute to correct expression in certain neuronal subtypes. These in vivo data imply that the regulatory elements located in the 5′-flanking region are capable of directing neuron-specific expression of the peripherin gene.

In order to identify the regulatory factors necessary for the cell-specific expression of peripherin, we have previously dissected its 5′-flanking sequence and localized a negative regulatory element (NRE) centered at −173 whose deletion results in elevated basal expression of the gene (1). In addition, there are two positive regulatory regions required for full induction in PC12 cells treated with NGF: a distal positive region approximately 2450 bp upstream of the transcription start site and a proximal constitutive region within 111 bp of the transcription start site (1). We proposed a two-step model of transcriptional activation of peripherin by NGF in which dissociation of a repressor from the protein complex at the NRE, coupled with a positive signal from the distal positive element, results in complete activation of the gene. We also showed that a multiprotein complex containing a member of the CTF/NF-1 family as the DNA-binding core protein interacts with the NRE (19). The composition of this complex changes after NGF treatment, coincident with derepression of the peripherin gene.

We have now finely mapped the distal positive element (DPE) necessary for the full expression of peripherin in NGF-treated PC12 cells. The isolated DPE is constitutively active in PC12 cells and retains significant activity in non-neuronal cells.
as well. We show that a distal negative element 5' to the DPE (between −3710 and −2660) may restrict the activity of the DPE in non-neuronal cells.

Here we present a detailed analysis of the minimal cis-acting sequences within the DPE that are necessary for its activity. Methylation interference footprinting of the DPE demonstrates two protein binding sites, a unique inverted repeat sequence as well as a sequence 38 bp upstream that resembles an Ets protein binding site. The Ets protooncogene family is a novel class of eukaryotic sequence-specific DNA-binding proteins (Ref. 20, reviewed in Refs. 21–23). The Ets proteins are related to each other based on sequence similarity in the ETS domain, which is the DNA binding domain. The Ets family proteins recognize purine-rich sequences characterized by an invariant GGA core sequence (23–25). Several members of the Ets family function as transcriptional activators, and a subset of these Ets family proteins are thought to require cooperation with other DNA-binding proteins for their activity (Ref. 26, reviewed in Refs. 21 and 23). In this report we present evidence that an Ets-related protein interacts with a novel inverted repeat binding protein to form a transcriptionally active complex at the DPE.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatment—Stock cultures of PC12 cells were maintained as described previously (27). PC12 cells grown for nuclear extract preparation were plated on plastic culture dishes coated with collagen (Celtrix Laboratories, Palo Alto, CA) at a density of 3 × 10⁵ cells/150-mm dish. NGF was added 24 h after plating (50 ng/ml; 2.5 S, Bioproducts for Science, Indianapolis, IN). NIH 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% defined and supplemented calf serum (HyClone, Logan, UT).

Nuclear Extract Preparation—Nuclear extracts were prepared from PC12 cells and NIH 3T3 cells according to the method of Dignam et al. (28). PC12 cells were cultured with NGF (50 ng/ml) for 95 days prior to harvesting, unless otherwise indicated.

Gel Retardation Assays (EMSA)—The conditions for the DNA-protein binding reactions were essentially as described previously (1). Competition experiments included 150-fold molar excess of unlabeled double-stranded oligonucleotides unless otherwise indicated. DNA-protein complexes were resolved on 5% or 4% polyacrylamide gels (30:0.8, double-stranded oligonucleotides unless otherwise indicated. DNA-protein binding reactions were essentially as described previously (1).

Fig. 1 summarizes the activities in PC12 cells in response to NGF (1). In order to define the minimal sequence necessary for activity, we used PCR technology to construct a series of peripherin-CAT reporter constructs with progressive deletions from −2660 to −2308 (Fig. 1B). The peripherin-CAT reporter constructs were transiently transfected into PC12 cells, which were subsequently treated with or without NGF. Fig. 1A summarizes their activities in PC12 cells in response to NGF. The −2660-CAT construct responds 5-fold to NGF and has 10-fold more activity than the −2308-CAT construct. There is a 7-fold drop in the activity of the −2308-CAT construct relative to the −2308-CAT construct in NGF-treated cells, defining a 26-bp sequence necessary for positive activity in response to NGF. The observation that a construct missing −2397 to −2308 (−2397 3′-CAT) responds less than 2-fold to NGF treatment confirms the importance of the region 3′ of −2396 containing the 26-bp element. The increase in activity upon deletion from −2660 to −2445 suggests the presence of an
of the 26-bp region (PositiveElement—Upon inspection, we noted that the sequence revealstworegions with GGAmotifs that form the invariant core of DNA binding sites recognized by Ets family proteins (23–25). One site is centered around nucleotide −2430, and the second site is immediately upstream from the 26-bp element, around −2402.

Methylation Interference Footprinting of the DPE Defines Two Protein Contact Sites—To determine where DNA-protein contacts occur within the DPE, we performed methylation interference footprinting with the 109-bp DPE probe. Fig. 3 demonstrates that two sites are contacted by proteins. First, probe molecules methylated at two Gs in the inverted repeat site and one G residue immediately 5' are underrepresented in the retarded DNA-protein complex. A second region of protein-DNA contacts involves the GGA motif rather than the immediately 3' GGAA, which is actually a more complete Ets protein core motif (see Fig. 3 and Refs. 23–25).

In order to confirm that formation of the specific DNA-protein complex depends just upon the inverted repeat site and the Ets-like site, EMSAs were performed using the same radiolabeled probe as in Fig. 2B (DPE; −2445 to −2337) and nuclear extracts from PC12 cells treated with NGF for 5 days. Competitor fragments identical in length to the DPE probe but containing mutated nucleotides in the Ets-like site, the inverted repeat site, or both sites were generated by PCR using mutant templates created by site-directed mutagenesis. Mutations introduced into the Ets-like site or the inverted repeat site were as listed for the mutant oligonucleotides (E−, I−) in Table I. Each nucleotide shown by methylation interference footprinting to contact protein was mutated; in addition, the 3' GGAA was mutated in the E− oligonucleotide and additional palin-
The Distal Positive Element of the Peripherin Gene

The sequence surrounding the footprinted GGA motif at nucleotide −2430 is similar to the consensus sites for Ets-1 and Ets-2 (22-25) in 6 out of 10 bp, five of which are contiguous and include the GGA core. To test the hypothesis that an Ets family member participates in the formation of the specific DNA-protein complex at the DPE, antibody competition assays were performed using a polyclonal anti-ETS domain antibody. This antibody was raised against a peptide in the highly conserved DNA binding domain (ETS domain) of Ets-1 and cross-reacts with most Ets proteins (Santa Cruz Biotechnology). The −2445 to −2337 radiolabeled DPE probe was incubated with nuclear extracts from differentiated PC12 cells in the presence or absence of antibodies. The left panel of Fig. 4 shows that formation of the DNA-protein complex is disrupted by incubation of extract with anti-ETS domain antibodies prior to addition of the DNA probe (Fig. 4A, lane 2). Two antibodies of unrelated specificity (anti-HMG and anti-interleukin 8), as well as pre-immune serum, have no effect on DNA-protein complex formation (Fig. 4B, lanes 2-4). A further control experiment in the right panel of Fig. 4 shows that the anti-ETS domain antibody has no effect on DNA-protein complex formation with an Sp1 probe, as expected. These results support the hypothesis that an Ets family protein binds to the DPE at a novel Ets-binding site.

Sequence Requirements for DNA-Protein Complex Formation—To determine the relative dependence of the formation of the specific DNA-protein complex on each site, EMSA was carried out using a series of mutant DPE probes of identical length. These DNA fragments, mutated in the Ets-like site, the inverted repeat site, or both sites, are identical to those used as competitors in Fig. 3B. Fig. 5 shows that probes containing mutations in both sites (E−I−) or in the inverted repeat site alone (E−I−) were unable to form the same DNA-protein complex as the wild type probe. The complexes formed (Fig. 5A, lanes 6 and 9) can be competed by unlabeled E+I− or E−I+ probe but not by wild type probe and thus may represent protein-DNA interactions at a site created by the mutagenesis. Unexpectedly, a probe (E−I+) containing the mutated Ets-like sequence and wild type inverted repeat sequence could generate a specific DNA-protein complex similar to the wild type probe (Fig. 5A, lane 3). However, on-rate analysis demonstrates that it takes up to 20 min or more for proteins to form a detectable complex with the E−I+ probe (Fig. 5B, lanes 5-8), whereas a complex forms in 5 min or less with wild type probe (Fig. 5B, lanes 1-4). In order to compare the migration of the DNA-protein complex formed on the wild type probe with the complex formed on the E−I+ probe, the EMSA was performed in 4% polyacrylamide gel and run for 3.5 h. Fig. 5C demon-

wild type DPE (E+I+); lane 3, wild type Ets-like site and mutated inverted repeat site (E−I−); lane 4, mutated Ets-like site and wild type inverted repeat site (E−I−); lane 5, mutations in both sites (E−I−). The specific DPE-protein complex is indicated with an arrowhead.
The Distal Positive Element of the Peripherin Gene

**TABLE I**

| Oligonucleotide                      | Sequence                                    |
|-------------------------------------|---------------------------------------------|
| Wild type Ets-like site (E+)        | TCAGCACAGCAGGAGAA<sup>a</sup>              |
| Mutant Ets-like site (E-)           | GTCAGCAAGATTCTGTTGTTAAG<sup>b</sup>         |
| Wild type inverted repeat site (I+) | AGGACCCTGCTCTCTCTCC                         |
| Mutant inverted repeat site (I-)    | AAGGAAGTGAAGATTCTGTTGTTAAG                 |

<sup>a</sup> Boldface letters indicate the DNA-protein contact points defined by methylation interference footprinting.

<sup>b</sup> Underlined nucleotides have been mutated from the peripherin sequence. Underlined and boldface nucleotides are mutations at the position of footprinted nucleotides.

*Fig. 4.* Evidence that an Ets protein binds to the Ets-like site in the DPE. EMSAs were performed with differentiated PC12 cell nuclear extract and 32P-labeled 109-bp DPE probe (panel A and panel B, lanes 1-4) or 32P-labeled Sp1 probe (panel B, lanes 5-6). Extract was preincubated with 2 μl of either anti-ETS-domain antibody (ETS; panel A, lane 2, and panel B, lane 6), pre-immune rabbit serum (pre panel B, lane 3), anti-HMG(Y) antibody (HMG; panel B, lane 2), or anti-interleukin 8 antibody (IL8; panel B, lane 4) for 1 h prior to the addition of the probe. Preincubation with an antibody to Ets protein resulted in a decrease in the intensity of the band formed with the Ets-like site probe and an increase in the intensity of the band formed with the Sp1 probe. These data suggest that the migration of the complex formed on both probes is similar; however, the intensity of the complex formed with the Ets-like site probe is less than that of the complex formed with the wild type probe after the same incubation period. Taken together, these data suggest that the inverted repeat site must be present for specific complex formation to occur. Moreover, the presence of the Ets-related protein may help stabilize the DNA-protein complex on the wild type probe.

To further investigate requirements for interaction of protein with the two sites in the DPE, an EMSA was performed using oligonucleotides containing a dimer of either site. Previously, we have observed that radiolabeled oligonucleotide probes containing a single copy of the inverted repeat site or the Ets-like site alone were unable to form a stable complex (data not shown). However, a stable complex does form on probes containing a dimer of either the Ets-like site or the inverted repeat site. Complex formation with each dimer probe (Fig. 6, lanes 1 and 6) is specifically competed by unlabeled oligonucleotides representing the wild type site (Fig. 6, lanes 2 and 7) but not by the corresponding mutant oligonucleotides (Fig. 6, lanes 3 and 8). Surprisingly, the complex formed with the inverted repeat site probe can be effectively competed by an Ets-like site oligonucleotide, and the complex formed with the Ets-like site can be effectively competed by an inverted repeat site oligonucleotide (Fig. 6, lanes 4 and 9). The corresponding mutant oligonucleotides (Fig. 6, lanes 5 and 10) do not compete significantly. In addition, complex formation is effectively competed by the 109-bp DPE fragment but not a DPE fragment mutated at both sites (data not shown). These data suggest that the DPE complex consists of two proteins that can be tethered at either site, leaving the DNA-binding site of the associated protein free to interact with competitor DNA.

*Sitedirected Mutagenesis of the DPE—*The distal positive region has been shown to be necessary for full activity of the peripherin promoter in response to NGF. In order to characterize the respective contribution of the inverted repeat site
and the Ets-like site to the activity of this region, site-directed mutagenesis of each sequence was performed in the context of the peripherin 5′-flanking region. Peripherin-CAT reporter constructs containing point mutations in the inverted repeat site (E−I−), the Ets-like site (E−I+) or both sites (E−I−) were transiently transfected into PC12 cells, which were subsequently treated with or without NGF. Fig. 7 summarizes the activity of wild type and mutant constructs in PC12 cells in response to NGF. Mutations in the inverted repeat site alone or both sites produced a 4.8-fold decrease in the activity of the DPE, whereas mutations in the Ets-like site reduced activity of the DPE 2.5-fold. These results correlate with the EMSA data (Fig. 5B) in which complex formation occurred with the E−I+ probe with a significantly slower on rate, but no complex formed with the E+I− or the E−I− probe. These data suggest that both the inverted repeat and the Ets-like sites are required for the full activity of the DPE.

In light of the data from the site-directed mutagenesis, it was initially perplexing that the activity of the −2396-CAT construct is comparable with that of the −2445-CAT construct, which contains the Ets-like site in addition to the inverted repeat site (Fig. 1). Careful inspection revealed a DNA sequence in the pUC-derived vector 5′ of the junction with the peripherin sequence, which matches the peripherin Ets-like site in five contiguous nucleotides out of eight comprising the Ets consensus sequence. Treisman et al. (42) report a similar artifactual result in which a plasmid-derived Elk-1/Sap-1 site interfered with an inserted SRF site. Since vector sequences can influence the promoter activity of test plasmids, we believe that examination of the relative activity of each site by site-directed mutagenesis in the context of an equal length of peripherin sequence yields the most accurate results.

The DPE Is Constitutively Active in PC12 Cells—To test whether the DPE can confer NGF responsiveness to a heterologous promoter, the isolated 109-bp DNA fragment (−2445 to −2337) containing the inverted repeat site and the Ets-like site was cloned 5′ of the TK promoter driving a CAT reporter gene (33). Inclusion of the DPE fragment 5′ of the TK promoter resulted in a 7-fold higher CAT activity than the TK promoter alone, whether or not the PC12 cells were treated with NGF (Fig. 8A). This result suggests that the DPE is acting as a constitutively positive regulator. The activity of the isolated DPE in undifferentiated PC12 cells is consistent with EMSA results demonstrating that proteins binding to the DPE are not NGF-inducible but function as constitutive positive acting transcription factors. In order to ascertain whether the DPE is active in non-neuronal cells as well as neuronal precursors (undifferentiated PC12 cells), we transfected the same peripherin-CAT constructs as described in Fig. 7 into 3T3 fibroblast cells. The activity of the wild type −2445-CAT construct is 3.2-fold higher than that of the −2308-CAT construct, whereas the activities of constructs containing mutations in the inverted repeat site, in the Ets-like site, or in both sites are similar to that of the −2308-CAT construct (data not shown). Therefore, the DPE is a positive element in 3T3 cells, and the effect of mutations at both the inverted repeat site and the Ets-like site on the activity of the DPE are similar in both 3T3 cells and PC12 cells. Again, these data correspond to the EMSA results, which indicate that a specific DNA-protein complex forms at the DPE with nuclear extracts from 3T3 cells (Fig. 8B, lane 5).

A Distal Negative Element Is Necessary for Tissue-specific Expression of the Peripherin Gene—Since our results demonstrate that there is significant activity of the −2445-CAT peripherin reporter construct in 3T3 cells, this suggests that the NRE element that represses peripherin activity in undifferentiated PC12 cells (1) is not sufficient to overcome the positive...
The Distal Positive Element of the Peripherin Gene

The DPE is constitutively active. A, a 109-bp DPE fragment containing the peripherin sequence (−2445 to −2337) was cloned into the TK-CAT vector pBLCAT2 (35). Equivalent amounts of plasmid DNA were co-transfected with 4 μg of RSV-β-galactosidase plasmid into PC12 cells by electroporation. Parallel plates were treated with or without NGF for 46 h prior to harvesting the cells. A representative CAT assay performed with protein extracts from the transfected cells is shown. B, EMSAs were performed using 32P-labeled DPE as a probe and nuclear extracts from undifferentiated PC12 cells (lanes 1 and 2), differentiated PC12 cells treated with NGF for 6 days (lanes 3 and 4), or 3T3 cells (lanes 5 and 6). 100-fold molar excess of unlabeled DPE DNA was included in lanes 2, 4, and 6 as competitor.

DISCUSSION

We have identified a DPE in the 5′-flanking region of the peripherin gene, which is required for the full induction of peripherin in PC12 cells. Methylation interference footprinting of the DPE identifies two DNA-protein binding sites separated by 38 bp: a novel inverted repeat site and an Ets-like site. Functional data based on site-directed mutagenesis of these two sequences demonstrates that the combination of the Ets-like site and the inverted repeat site is necessary for significant activity of the DPE. Our results suggest that both the Ets-like site binding protein and the inverted repeat binding protein form a ternary complex at the DPE and act synergistically to achieve efficient activation of the peripherin promoter. There is a growing list of positive elements in which synergy exists between an Ets family member and a transcription factor of another class that binds to an adjacent site. This list includes Ets-1 and Sp1 at the HTLV-1 LTR (43), PU.1 and NF-EM5 at the immunoglobulin κ3 enhancer (44) and the immunoglobulin λ2–4 enhancer (45), Ets-2 and Myb at the mim-1 promoter (46), and Elk-1 or Sap-1 and SRF at the serum response element of the fos promoter (47, 48). The nature of the interactions among the Ets proteins, the adjacent transcription factors, and their cognate DNA binding sites is variable (reviewed in Refs. 21 and 23).

There are several novel features of the Ets-related protein interaction at the peripherin DPE. First, the Ets-like site and the inverted repeat site are separated by 38 bp. In the above listed examples, the location of the Ets site is only 1–3 nucleotides away from the site of its synergistic partner. Treisman et al. (42) have shown that ternary complex formation between Elk-1 and SRF occurs on synthetic oligonucleotides in which spacing between the sites varies from the wild type (2 bp) to as far as 27 bp, suggesting that there is flexibility in the spacing requirements for ternary complex formation. Our data provide evidence for a functional interaction between an Ets protein and a transcription factor that binds to a site that is not directly adjacent.

The second novel feature of the Ets protein interaction at the DPE is that the Ets-like site (AGGAG) deviates from the Ets canonical recognition motif (CA)GGGA(T/A)) at the fifth position (22–25). However, EMSAs using an anti-ETS domain antibody confirm that the DNA-protein complex formed at the DPE contains an Ets family member. It has been proposed that the Ets protein interaction with another transcription factor may influence its ability to bind preferentially to a subset of possible Ets sites (reviewed in Ref. 21). Therefore, interaction with the inverted repeat protein at the DPE may result in the binding of the Ets-like protein to a noncanonical site.

The third novel feature of the ternary complex is that the inverted repeat sequence does not match any known sites in the Transcription Factors Database (TFD7.3). We have utilized the yeast one-hybrid screen to obtain candidate cDNA clones from a rat olfactory epithelium cDNA library (generously donated by Dr. R. Reed, Johns Hopkins University; Ref. 49) that will activate a reporter plasmid via multiple inverted repeat sites.

Our data demonstrate that the proteins binding to the Ets-like site and the inverted repeat site interact to form a specific DNA-protein complex at the DPE. We have shown that specific complex formation still occurs on a DPE probe containing the mutated Ets-like site and the wild type inverted repeat site (E−1+). However, the complex formed on this mutated probe behaves differently from the one on the wild type DPE. The on rate of complex formation on the mutated DPE is 4-fold slower than that of complex formation on the wild type DPE (See Fig. 5B). The relative rate of complex formation reflects the activation energy that must be overcome for the DNA-protein interaction to occur, especially if a conformational change must occur (50). In addition, in vivo experiments indicate that the complex on the mutated DPE is less active in transactivation of the peripherin promoter. Taken together, these data suggest that when the Ets-related protein and the inverted repeat binding protein both interact with DNA in the ternary complex, the activation energy is reduced due to formation of a more
The Distal Positive Element of the Peripherin Gene

stable complex. This results in increased transcriptional activity of the DPE.

Our data suggest that the Ets-related protein cannot bind to the DPE autonomously. A subset of Ets family proteins that display little or no independent DNA binding (e.g. Ets-1 and Ets-2 (26, 51) and Elk-1 or Sap-1 (42, 47, 52)) contain an inhibitory domain that may hinder interaction with DNA. However, this inhibition may be relieved by interaction with an adjacent factor. For example, Elk-1 cannot bind independently to the Ets site at the serum response element but rather requires the presence of SRF at an adjacent site (24, 48, 53). It will be interesting to determine whether the Ets-related protein binding at the DPE contains a similar inhibitory domain.

We have presented indirect evidence that the Ets-related protein may interact with the inverted repeat binding protein through protein-protein interactions (See Fig. 6). The protein complex formed on a dimer of either the Ets-like site or the inverted repeat site can be specifically competed by excess unlabeled competitor oligonucleotides containing either binding site. This cross-competition suggests that the Ets-related protein and the inverted repeat protein may be tethered together and potentially interact with either the Ets sequence or the inverted repeat sequence via the DNA binding domain of the appropriate member of the complex. The other DNA binding domain would be free to be contacted by competitor DNA. This model predicts that the Ets-related protein is present in the DNA-protein complex formed on the mutated DPE (E−1+), thereby explaining the similar migration of the complex formed on the wild type DPE and the mutated DPE (See Fig. 5C).

Based on our findings, we propose a mechanism of ternary complex formation at the DPE in which the Ets-related protein cannot bind to the Ets-like site autonomously but rather is recruited to the DPE by interaction with the inverted repeat protein. Formation of this ternary complex in which the Ets-related protein and inverted repeat protein both interact with DNA, as well as each other, results in a stable, transcriptionally active DNA-protein complex.

Finally, we have found that the DPE is not an NGF response element. It is constitutively active in PC12 cells, as shown by the activity of the DPE-TK-CAT reporter transfected into cells with and without subsequent treatment with NGF. In addition, it is moderately active in 3T3 cells. In light of these results, how does one explain the NGF-inducible and tissue-specific pattern of expression of the peripherin gene? This specific pattern of expression appears to be the result of the action of two separate negative regulatory elements in the 5′-flanking region modulating the constitutive activity of the DPE: the proximal negative element (NRE, −173) and the distal negative element (DNR, −3710 to −2660). Several results suggest that the proximal NRE rather than the DNR is able to repress DPE activity in 3T3 cells (see Fig. 9). Therefore, the relative activity of each negative element may differ in neurogenic versus non-neuronal cells. We are currently testing the relative in vivo importance of the NRE and the DNR in transgenic mice.

The regulation of neural-specific gene expression by the interaction of negative regulatory elements with a constitutively positive element is in keeping with the hypothesis that the neural state is the "default" state in development (54, 55). Several neural-specific structural genes, including SCG10 (56–58), the type II sodium channel gene (59), and dopamine β-hydroxylase (60) have constitutively active promoter/ enhancers whose expression is regulated by a upstream silencer element (reviewed in Ref. 61). The silencer element in SCG10 and the type II sodium channel gene is bound by a protein expressed in both non-neuronal cells and neuronal precursors (62, 63). A unique feature of peripherin gene regulation may be the differential effect of the two negative elements in different cell types.

This report is the first to demonstrate a functional role for an Ets protein in the activation of a mammalian neural-specific structural gene. Interestingly, an Ets-like recognition sequence has been noted in the NF-L promoter (64). Elucidation of the mechanism by which the ternary complex at the DPE activates transcription should reveal how its action is modulated by proximal and distal repressors, resulting in neural-specific expression.

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