The TATA Motif Is a Target for Efficient Transcriptional Activation and Nerve Growth Factor Induction of the Peripherin Gene*

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Three proximal elements, PER1, PER2, and PER3, have been implicated in the regulation of peripherin gene expression. PER1 contains the TATA motif and was identified as the principal mediator of neuronal specificity. Here, we demonstrate by transfection of constructs mutated in PER1 that the in vitro protein binding activity of PER1 is irrelevant to its function. However, mutations or substitutions in the TATA box decreased promoter activity by up to 80%. We have investigated this unusual preference for a particular TATA sequence in PC12 cells. In these cells, nerve growth factor induces neuronal differentiation, increasing peripherin gene expression 3-4-fold, while dexamethasone elicits chromaffin differentiation and a 3-fold decrease in peripherin mRNA. Experiments with stably transfected PC12 cells revealed that the specific TATA box of the peripherin gene was crucial for nerve growth factor response. However, it did not affect dexamethasone down-regulation. Therefore, nerve growth factor acts through an element essential for neuronal peripherin gene expression. The results predict that proteins interacting in the vicinity of the TATA box, by inference factors associated with the preinitiation complex, are important for peripherin gene regulation and provide new insights into the mechanisms underlying neuronal differentiation.

The regulation of genes expressed exclusively in neurons is not well understood. A large number of neuronal regulatory genes have been identified but little is known about their targets and their roles in differentiation of particular types of neurons. To study this problem, we have chosen peripherin, a type III intermediate filament protein (1). The peripherin gene provides several advantages for studies of neural specific gene expression. Like most other IF proteins, its expression is both tissue-specific and developmentally regulated (for a review, see Ref. 2). In contrast to the widespread nervous system distribution of the neurofilament triplet proteins, peripherin shows a more restricted distribution pattern (for a review, see Ref. 3). Peripherin is found in peripheral nervous system neurons, most cranial nerves, ventral horn motor neurons, and a few other nuclei in the central nervous system (4–7). During neuronal development, peripherin expression was never detected in regions of high mitotic activity or along routes of migration. Peripherin mRNA or protein were seen only in cells that had already migrated to their final position and were beginning to elaborate processes (8–10). Peripherin is also significantly up-regulated in response to nerve injury (11, 12). Therefore, peripherin is one constituent of a program of gene expression activated at terminal neuronal differentiation and possibly involved in axonal growth and regeneration.

Peripherin is also expressed in neuronal cell lines as well as in PC12 cells (13, 14). PC12 cells, originally derived from a rat pheochromocytoma (15), resemble neural crest-derived sympathetic precursor cells, their normal counterparts. Like sympathoadrenal precursors (16, 17), PC12 cells can display properties of neuronal or chromaffin cells depending on the environment (18). In the presence of nerve growth factor (NGF),1 these cells exhibit properties of sympathetic neurons, including neurites and the enhanced expression of various neuronal genes like peripherin (19, 20). By contrast, in the presence of glucocorticoids, the cells differentiate into adrenal chromaffin-like cells and neural specific genes are down-regulated (20–23). While several transcriptional changes are likely to be necessary to elicit these responses, the peripherin gene is one of the ultimate targets of NGF and glucocorticoids in that system. Studies of the regulation of peripherin gene transcription are therefore likely to lead to the identification of components of the signaling pathway involved in the implementation of the transcriptional changes necessary for differentiation.

Recently, by transfection and footprinting experiments, we have identified in the proximal promoter three regulatory elements important for peripherin gene expression, which we named PER1, PER2, and PER3 (24). PER2 and PER3 function as activators but have no effect on cell type specificity of expression. The PER3 element is a stronger activator than PER2, and mutation of PER3 in a construct containing the first 256 bp of the peripherin gene promoter severely affects reporter gene expression (25). We have shown that the GC-rich PER3 element binds the Sp1 transcription factor in vitro and in vivo and stimulates transcription when combined with PER1 (25). The PER1 element, which overlaps the TATA box, interacts with a protein-binding complex prevailing in peripherin-expressing cell lines and appears to be an important determinant of cell type specificity. Although this element by itself is inefficient in driving reporter gene expression, its fusion with the polyoma virus enhancer results in high transcriptional activity, but only in peripherin expressing cell lines (24). These experiments demonstrated that neuronal regulation of the peripherin gene

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1 The abbreviations used are: NGF, nerve growth factor; CAT, chloramphenicol acetyltransferase; β-gal, β-galactosidase; EMSA, electrophoretic mobility shift assay; bp, base pair(s).
is achieved through the interaction of its promoter with multiple DNA-binding proteins consisting of neuron-specific and ubiquitous activators.

To gain a better understanding of the mechanisms that control peripherin gene expression, we focused our studies on the PER1 element. Here, we report that the properties of the PER1 element depend primarily on the TATA box and to a lesser extent on the surrounding sequences. We also found that the response of the peripherin gene to NGF is mediated through the TATA box, while the response to glucocorticoids is not. These results identify the TATA box and the complex that is assembled there for transcription initiation as essential elements in the regulation of neuronal gene expression and the response to NGF.

**EXPERIMENTAL PROCEDURES**

**Plasmids—** Plasmids pGAL4350, pGAL425, pGAL256, and pGAL46 have been described previously (24). Mutagenesis was performed as described by Io et al. (26) on a 127-bp PstI fragment (−130/−3) cloned into the PstI site of pBluescript. After polymerase chain reaction mutagenesis, the mutated fragment Styl-PstI (−101/−3) was inserted in the pGAL256 or pGAL425 constructs. The oligonucleotides used for directed mutagenesis are shown in Table I.

Plasmid pGAL256T19 was constructed in two steps. First, the keratin 19 (K19) gene core promoter (−34/+55) obtained by digestion with Smal and BamHI was inserted between the Smal-BamHI polylinker sites of pG6D (24) after blunting the incompatible overhangs with Escherichia coli DNA polymerase I Klenow fragment, generating pGAL19. Then, pGAL256T19 was created by ligation of the following fragments into pGAL256 previously digested with Styl and KpnI: i) the K19 gene core promoter plus a portion of the lacZ gene isolated from pGALT19, containing an EcoRI site (end-blunted) at the 5’ end and a KpnI site at the 3’ end and ii) a portion of the peripherin gene promoter extending from HpaII to StyI (−46/−101) isolated from pGAL256. In the resulting plasmid, pGAL256T19, the peripherin gene core promoter was substituted by the K19 gene core promoter while the distance between PER1 and the TATA box was conserved. The plasmids used in transfection experiments were purified by alkali lysis and two rounds of CsCl density gradient sedimentation (27).

**Cell Culture and DNA Transfection—** The N18TG2 and OBL24 cell lines were cultured and seeded as described (24). N18TG2 is a peripherin-expressing cell line derived from a mouse neuroblastoma (28). OBL24 is a cell line that does not express peripherin. It was derived from mouse olfactory bulb cells immortalized by the avian myc oncogene (29). Calcium phosphate-DNA precipitates containing 15 μg of each peripherin-lacZ reporter plasmid and 5 μg of pSV2CAT to monitor transfection efficiency were prepared as described before (24) except that CAT activities were quantitated by PhosphorImager analysis (28). The data presented are the averages of at least four transfections done in duplicate.

PC12 cells (ATCC CRL 1721) were grown on collagen-coated culture dishes in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum. To produce stable lines containing the various lacZ reporter constructs, PC12 cells were transfected to a density of 2 × 10^5 cells/6-cm dish. Six hours later, the cells were transfected by the calcium phosphate method with 20 μg of peripherin-lacZ reporter plasmids and 2 μg of pRVSneo (ATCC T7198) as described for transient transfection and glyceraldehyde-3-phosphate dehydrogenase (Sigma) for 7 days. β-Galactosidase activities were measured and normalized on total cell protein concentration.

**Northern Analysis—** Total RNA was isolated from PC12 cells, NGF-treated PC12 cells, and demethasone-treated PC12 cells by LiCl precipitation (31). 10 or 20 μg of total RNA were fractionated on 2.2 M formaldehyde-agarose gel and transferred onto nylon membrane by blotting in 20 × SSC (3 M NaCl, 0.3 M sodium citrate, pH 7). The 32P-labeled peripherin probe was hybridized for 18 h in 50% formamide, 5 × SSC, 0.1 M sodium phosphate, pH 7.0, 0.5% SDS, 0.1% non-detergent milk, 10% dextran sulfate, and 200 μg/ml herring sperm DNA at 42 °C. The peripherin probe was prepared by random primed labeling of excised SpHISmal fragment (−256/−142) of the mouse peripherin gene. Blots were washed at 55 °C for successive 20-min periods in 2 × SSC and 0.2 × SSC in the presence of 0.1% SDS. Blots were exposed to XRP-1 film with intensifying screens at −70 °C. Quantitative analysis of total RNA was done by methylene blue staining as described (32). Peripherin mRNA was quantitated by PhosphorImager analysis. Electrophoretic Mobility Shift Assays—Nuclear extract proteins from N18TG2, PC12, and NGF-treated (72 h) PC12 cells were prepared essentially by the method of Dignam et al. (33). The 127-bp fragment (−130/−3) and derived mutant fragments subcloned into the PstI site of pBluescript were cut with EcoRI and PstI-end-labeled with [γ-32P]ATP using E. coli DNA polymerase I Klenow fragment. The 42-bp DNA fragment was purified by 8% polyacrylamide gel electrophoresis after digestion with HpaII and CiaI double digestion. PER1 (formerly TAI, Ref. 24) was also used. Oligonucleotides were labeled with [γ-32P]ATP and T4 polynucleotide kinase and purified from unincorporated radioactivity by 8% polyacrylamide gel electrophoresis. Electrophoretic mobility shift assays (EMSA) were performed as described (24) except that 2.5 μg of poly(dI-dC) were used.

**RESULTS**

**Role of PER1 in the Regulation of Peripherin Gene Expression—** To characterize PER1, we introduced mutations in its sequence and determined their effects on the capacity of PER1 to interact with proteins by EMSA and on reporter gene activity after insertion of the mutations into pGAL256, a lacZ reporter gene construct with 256 bp of peripherin gene promoter sequence (24), and transient transfections. Of eight mutations examined, three, PER1M2, PER1M5 and PER1M6, affected neither the capacity of PER1 to form a complex with nuclear proteins in gel shift assays (Fig. 1A) nor reporter gene activity in transfected N18TG2 cells (Fig. 1B). PER1M4 and PER1M7, two mutations which abrogate the in vitro protein binding capacity of PER1 (Fig. 1A), resulted in a 40–50% reduction of reporter gene activity in N18TG2 cells (Fig. 1B). However, two other mutations, which also abrogate the protein binding capacity of PER1, PER1M1, and PER1M3 (Fig. 1A), had no effect on reporter gene expression when inserted into pGAL256 (Fig. 1B). The last mutation examined, PER1M10, which by EMSA does not interfere with the binding of proteins to PER1 (Fig. 1A), resulted in an 80% reduction of β-galactosidase activity when compared with the wild type promoter construct (Fig. 1B). Finally, when the eight mutant constructs were transfected into OBL24 cells, which do not express the peripherin gene, none of them resulted in significant reporter gene expression (data not shown). Three conclusions can be drawn from these experiments: (a) cell type specificity is affected neither by mutations which abrogate protein binding to PER1 nor by mutations which reduce promoter strength, (b) the loss of the capacity to bind proteins does not correlate with promoter strength in peripherin expressing cells, and (c) mutations that result in de-
creased reporter gene activity localize to a short sequence centered on the TATA box of the gene (see Fig. 1A, shaded area), suggesting that this sequence plays a regulatory role.

**Role of the TATA Box Sequence for Promoter Activity and Cell Type Specificity**—To further characterize the relationship between gene expression and the TATA box sequence, we compared the efficiency of various TATA elements by transient transfections. When the peripherin gene TATAAA sequence was mutated to the SV40 early promoter TATA box sequence (TTTTATTTAT) in a 256-bp peripherin-lacZ construct and transfected into N18TG2 cells, the activity of the resulting plasmid, pGAL256Tsv40, was only 17% of the wild type construct (Fig. 2). Similarly, conversion of the peripherin gene core promoter to the keratin 19 gene core promoter (with TATA box sequence ATAAAAA) or to the HSV-1 thymidine kinase gene core promoter (with TATA box sequence ATATTAA) resulted in two plasmids, pGAL256T19 and pGAL256Ttk, that were, respectively, only 18 and 35% as active as the wild type construct (Fig. 2). Last, mutation of the TATA sequence of the peripherin gene to a nonsense control sequence (TCGA) in plasmid pGAL256M8 resulted in a reduction of reporter gene expression to 20% (Fig. 2). Thus, changes in the TATA box sequence decreased gene expression by as much as 80% relative to the wild-type promoter. However, from the difference in efficiency between mutant M4 (see Fig. 1), which reconstitutes the wild-type promoter. However, from the difference in efficiency between mutant M4 (see Fig. 1), which reconstitutes the wild-type promoter, to the mixture prior to probe addition. The PER1M1 and PER1M4 probes have weak binding activity that could not be competed by PER1 (data not shown). B, pGAL256 and derived mutated constructs were transiently transfected into N18TG2 cells. β-Gal activities have been normalized to the pSV2CAT control and are expressed relative to the activity of pGAL256 set at 100%.

was therefore of interest to determine if the core promoter and in particular the TATA box sequence played a role in this response. To address this question, we tested the ability of peripherin gene promoter constructs with mutations in the core promoter to up-regulate reporter gene expression in PC12 cells following NGF treatment. First, we measured the increase of peripherin mRNA levels following NGF treatment by Northern analysis. Peripherin mRNA levels increased markedly with long term NGF treatment (Fig. 3A). The level of peripherin mRNA in PC12 cells treated with NGF for 72 h was about 3.5-fold higher than in untreated cells, which corresponds to the increase reported by Leonard et al. (20). Second, constructs containing the peripherin gene promoter linked to the lacZ reporter gene were stably transfected into PC12 cells. After neomycin selection, colonies were pooled, maintained as polyclonal cell lines, and subsequently treated or not with NGF for 72 h. In cell lines carrying constructs pGAL3450, pGAL425, and pGAL256, addition of NGF increased reporter gene activity by 4.4-, 3.0-, and 2.1-fold, respectively (Table II). The β-gal activity was also increased 2.3-fold by NGF in cells containing pGAL256M1 (Table II), a mutant affected in the capacity of the PER1 element to bind proteins in vitro, but with an intact TATA sequence (see Fig. 1A). By contrast, pGAL256M4, which contains the HSV-1 thymidine kinase TATA box sequence ATATTAA (see Fig. 1A), was not inducible by NGF (Table II). To confirm these results, the PER1M4 mutation was also introduced in pGAL425 to generate pGAL425M4. Contrary to pGAL425, pGAL425M4 expression was not stimulated by NGF (Table II). Furthermore, no induction by NGF was apparent in cells containing pGAL256T19 and pGAL256Tsv40, which contain, respectively, the keratin 19 gene core promoter and the TATA box of the SV40 early promoter (Table II). These results indicate clearly that core promoter mutations that alter the peripherin gene TATA box sequence abolish the response to NGF. Finally, to determine if the NGF response was associated with qualitative changes in PER1 binding activity, we performed EMSA with nuclear extract proteins from untreated and NGF-treated (72 h) PC12 cells. Similar binding patterns were observed with both nuclear extracts (Fig. 4). Thus, NGF treatment does not lead to the appearance of new nucleoprotein complexes or to modifications of the preexisting complex observed with PER1.

**Role of the TATA Box in the Response to Dexamethasone**—Neuronal specific genes are generally down-regulated by dexamethasone in PC12 cells. We were therefore interested to determine if peripherin mRNA was affected by dexamethasone
during PC12 differentiation and if so, what role, if any, the TATA box sequence might play in that process. As shown in Fig. 3B, peripherin mRNA levels were reduced 3.4-fold after 48 h of dexamethasone treatment. Using the stably transfected cell lines described above, we asked whether the 5'-flanking region of the peripherin gene promoter was sufficient to confer dexamethasone responsiveness. To answer this question, we treated the cell lines containing pGAL3450 and pGAL256 with dexamethasone for 7 days. The β-gal activities were reduced by about 3-fold in these cells (Table III), approximating the reduction in endogenous peripherin mRNA abundance observed by Northern analysis. Thus, most or all of the sequences necessary for mediating dexamethasone response appear to reside within the first 256 bp of the peripherin gene promoter. We then determined if mutations in the TATA box could affect the response to dexamethasone. As shown in Table III, β-gal activities observed with cell lines containing pGAL256M1, pGAL256M4, and pGAL256T19 were down-regulated by dexamethasone. The only exception were the pGAL256Tsv40 containing cells where the β-gal activity did not seem to be affected by the treatment. These results suggest that the peripherin gene TATA box sequence is not involved in the down-regulation of peripherin gene transcription that occurs during dexamethasone-induced differentiation in PC12 cells.

**DISCUSSION**

Although a picture of peripherin gene transcriptional regulation is now emerging (19, 24, 25, 34, 35), the mechanisms directing its expression in specific cell types are still not well understood. In a previous study, we identified in the core promoter an element called PER1 that was sufficient to restrict the expression of a reporter gene to peripherin-expressing cells (24). Here, we show that the functional element within PER1 is the TATA box and that in addition to its role in conferring cell type specificity to the promoter, it mediates the response to NGF. These results predict that proteins interacting in the vicinity of the TATA box, by inference the preinitiation complex proteins, play an important regulatory role for peripherin gene expression.

There are several indications that transcriptional regulation can be mediated through the TATA box (36–39), and in a number of genes expressed in the nervous system (40–44), a
Our results indicate that, in addition to the TATA box, other regions of the peripherin gene promoter are involved in regulating the response to NGF. Reporter gene expression increases by 2–4-fold following NGF addition, approximating the peripheral gene response to NGF. Reduction of the promoters, which do not respond as much as pGAL3450 and pGAL425, suggesting the presence of other more distal elements contributing to the NGF response. Thompson et al. (19), studying the response of the rat peripherin gene, have suggested that NGF activates transcription in part by relieving the repression mediated by a negative response element in the promoter (19). However, the mouse and rat genes clearly differ in this respect, since we found no evidence for such an activity in the mouse peripherin gene (this study and Ref. 24). By contrast, the effect of glucocorticoids did involve a repressor element localized in the proximal promoter. Since we found no evidence for a glucocorticoid response element in that part of the promoter, the identity and location of the sequence that mediates the response to dexamethasone will require further studies.

In conclusion, the experiments described here provide evidence that the TATA box sequence of the peripheral gene plays an important role in the regulation of its transcription and suggest that the components assembled at the core promoter influence gene expression during differentiation. Elucidation of the molecular basis for cell type specificity and NGF inducibility of the peripheral gene will require a fine analysis of the molecules associated with the transcription initiation complex.

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short region of the promoter centered on the site of assembly of the preinitiation complex was found to exhibit substantial cell type-specific expression. In addition, there are several indications that specialized TATA sequences can play a role in the regulation of transcriptional induction (45–48). In the specific case of the response of the peripherin gene to NGF, two general mechanisms can be envisaged to explain how it may be affected by changes in the TATA box sequence. First, the TATA element and adjacent sequences can influence either TATA-binding protein or TFIIID binding affinity, promoter recognition by RNA polymerase II, and the general initiation factors or the topology of the preinitiation complex (49–52). Such changes could interfere with protein-protein interactions in the complex or between the complex and co-activators and lock it in a nonresponsive state.

A second possibility is that diverse TATA box elements could mediate the assembly of distinct preinitiation complexes; this would profoundly influence the response of a promoter to different activators. The existence of numerous TATA-binding protein associated factors (53–59) and the isolation of TFIIH subpopulations (60–62) and of functionally distinct TFIIH complexes (63) indicates that TATA-binding protein can be associated with distinct factors or sets of factors. The preference of a specific TFIIH complex for a particular TATA box sequence would effectively target the NGF response to a specific set of genes. Since the integrity of the TATA box of the peripherin gene is essential both for a high level of transcriptional activity and for the capacity to respond to NGF, we suggest that the effects observed with variant and nonconsensus TATA box sequences are more likely to result from interference with the assembly or with the function of a specific multiprotein complex rather than simply from changes in TATA-binding protein or TFIIH binding affinity.
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