Cloning and Characterization of the Promoter Region of the Rat Epidermal Growth Factor Receptor Gene and Its Transcriptional Regulation by Nerve Growth Factor in PC12 Cells*

(Received for publication, April 21, 1999, and in revised form, December 8, 1999)

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Our previous studies have shown that treatment of PC12 cells with nerve growth factor (NGF) causes a profound down-regulation of the epidermal growth factor receptor (EGFR) mRNA and protein. Further, the NGF-induced down-regulation of the EGFR is under transcriptional control. To elucidate the molecular mechanism of this down-regulation we have cloned a 2.7-kilobase sequence from the promoter region of the rat EGFR from a rat P1 library. Six transcriptional start sites were identified by 5′-rapid amplification of cDNA ends and primer extension. Sequence analysis showed a 62% overall homology with the human EGFR promoter region. To investigate its transcription, 1.1 kilobases of the 5′-flanking sequence were fused to a luciferase reporter gene. This sequence exhibited functional promoter activity in transient transfection experiments with PC12, C6, and CV-1 cells. Treatment of PC12 cells with NGF inhibited promoter activity. By transfection of promoter deletion constructs, a silencer element was found between nucleotides −280 and −181, and TCC repeat sequences appeared to be at least partially responsible for the down-regulation of the EGFR by NGF. Supportive evidence for the relevance of this sequence was obtained from gel mobility shift assays and by transfection of TCC mutation constructs. Our results demonstrate that TCC repeat sequences are required for the down-regulation of rat EGFR by NGF in PC12 cells and may lead to the identification of the NGF-responsive transcription factors.

Human epidermal growth factor (EGF)† is expressed in many tissue and cell types (1–4), and receptors (EGFR) on the cell surface are regulated by a variety of mechanisms. A relationship between the number of EGFR on the cell surface and tumorigenesis has been proposed (5). Overexpression of EGFR transcripts in a variety of tumors, such as those of the ovary, the cervix, and the kidney, results from transcriptional as well as posttranscriptional mechanisms (6). A variety of agents have been shown to increase EGFR gene expression (7–9). Repression of EGFR gene transcription by different agents has also been reported (10, 11). The mechanisms underlying the regulation of EGFR expression have not been defined completely, but transcriptional control appears to play a major role in this regulation.

The PC12 cell line is a clone derived from a pheochromocytoma tumor of the rat adrenal medulla which has become the premier model for the study of the action of nerve growth factor (NGF) (12, 13). These cells stop dividing and differentiate morphologically and biochemically into sympathetic neuron-like cells when treated with NGF. Before treatment, PC12 cells express plasma membrane receptors for EGF, which is a mild mitogen for them (14, 15). Thus, PC12 cells display receptors for both NGF, which stops them from dividing (14), and for EGF, which encourages them to divide (15). EGFR are down-regulated in PC12 cells upon treatment with NGF (14). We have suggested that this a mechanism by which NGF instructs PC12 cells to stop dividing and differentiate.

Our previous studies (16, 17) have shown that treatment of PC12 cells with NGF causes a profound down-regulation of both EGFR mRNA and protein. NGF-induced down-regulation of the EGFR is under transcriptional control (17), and that control is p140trk-, Ras-, and Src-dependent (16). The detailed cellular and molecular mechanisms that mediate NGF-induced EGFR down-regulation are unknown. Recent advances in the understanding of the signaling pathways activated by NGF receptors make PC12 cells a useful model for the study of cross-regulation among differentiating agents, such as NGF, and mitogens, such as EGF, during neuronal differentiation.

To study the molecular mechanisms of the down-regulation of the EGFR during the differentiation of PC12 cells, a rat cell line, it seemed reasonable to clone the promoter region of the rat EGFR from a rat P1 library. In this study, 2.7 kbp of the promoter region of the rat EGFR have been characterized. Six transcriptional start sites have been identified by 5′-rapid amplification of cDNA ends (RACE) and primer extension. We have characterized the receptor gene promoter by deletion and
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mutation analysis using an in vivo transfection assay. Finally, we have identified TCC repeat sequences of the promoter region that are at least partially responsible for the down-regulation of the EGFR by NGF during the differentiation of PC12 cells.

EXPERIMENTAL PROCEDURES

Materials—Mouse NGF and rat type I collagen were purchased from Becton Dickinson (Bedford, MA). LipofectAMINE was a product of Life Technologies, Inc.

Cell Culture—PC12 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, 10% horse serum, 100 μg of streptomycin/ml, and 100 units of penicillin/ml. For NGF treatment, 100 ng of NGF/ml was added to the culture medium every other day. African green monkey kidney cells (CV-1) or C6 glioma cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 μg of streptomycin/ml, and 100 units of penicillin/ml. In all experiments, cells were cultured on collagen. Collagen coating of culture dishes and flasks was performed according to the manufacturer’s protocol (5 μg/cm²).

Genomic Cloning, Southern Blot Analysis, and Sequence Comparison—To clone the rat EGFR 5′-flanking region, the following two primers were used: 5′-GGACGCACACGAAGACAGGC-3′, which spanned nucleotides 1–20 of rat EGFR cDNA sequence, and 5′-GGACCGCCACCAAGACAGGC-3′, which is complementary to nucleotides 134–155 of the cDNA sequence (19). The PCR product amplified from rat genomic DNA using the above two primers was sequenced. The primers were then used in a PCR screen to identify bacteriophage P1 clones encoding the rat EGFR 5′-flanking region (custom service provided by Genome Systems, Inc., St. Louis, MO). The PCR profile included denaturation at 94 °C for 1 min, primer annealing at 62 °C for 1 min, and primer extension at 72 °C for 30 s, with a final concentration of MgCl₂ of 1 mM. One P1 clone named GS17714 encoding the rat EGFR gene was obtained using PCR screening. Two DNA restriction fragments, named pBS1 and pBS2 (see Fig. 1), derived from the GS17714 clone, were subcloned into pBluescript II KS(−) (Stratagene) for restriction enzyme mapping and DNA sequencing. Southern blot analysis of rat genomic DNA with a random labeled probe spanning nucleotides 1–155 of rat EGFR cDNA sequence was performed as described (18). The clones were sequenced with an Applied Biosystems model 373A automated DNA sequencer. Sequence comparison was carried out using the Blast and Bestfit programs from Genetics Computer Group (Madison, WI).

Primer Extension Analysis—Primer extension was carried out as described previously (19). An antisense nucleotide (5′-AGCAGTAGCTTGTTTCTGCCAG-3′) complementary to nucleotides 170–191 of the rat EGFR cDNA sequence was radioabeled at the 5′-end with T4 polynucleotide kinase and [γ-³²P]ATP (3,000 Ci/mmol, Amersham Pharmacia Biotech). The radioabeled primer (15 ng) was added to 10 μg of poly(A)⁺ RNA isolated from either PC12 or C6 cells in 20 μl of hybridization buffer (0.6 M NaCl, 0.01 M Tris-HCl, pH 8.3, 0.01 M EDTA, 0.5 mM spermidine, 1 mM dNTPs, and 0.5 mM spermine) and the reaction was carried out at 42 °C for 1 h. After completion of the reaction, samples were extracted with phenol/chloroform and precipitated with ethanol. The extension products were digested in a denaturing dye solution and analyzed on a 6% polyacrylamide-urea gel. The size was determined by comparison with a DNA sequencing ladder. Standards for sizing primer extension products were generated from the control sequence of the Sequenase version 2 kit (U. S. Biochemical Corp.). These were synthesized according to the manufacturer’s instructions with [γ-³²P]ATP using single-stranded M13mp18 DNA and the T4 polynucleotide kinase and the 3′-90 primer provided.

RACE—5′ RACE-Ready cDNA (rat kidney) was purchased from CLONTECH (Palo Alto, CA). Rat EGFR-specific primers refg1235A (5′-GCCGACACGCCGAGAGAGAACACTGATG-3′) and refg1235B (5′-AGCAGTAGCTTGTTTCTGCCAG-3′) were reverse transcription-PCR amplified from rat EGFR mRNA using the Prime program from Genetics Computer Group. PCR parameters were 94 °C for 30 s, 94 °C for 5 s, 94 °C for 5 s, 72 °C for 3 min for 5 cycles 94 °C for 5 s and 70 °C for 3 min for 5 cycles, 94 °C for 5 s, and 68 °C for 5 min for 25 cycles. Major bands of about 1.4–1.6 kbp were obtained after amplification, purified by agarose gel electrophoresis, and cloned into the pCR II vector (In-vitrogen). 30 recombinant clones were picked for subsequent sequence analysis.

Construction of Plasmids for Promoter Analysis—To analyze the active promoter region of the rat EGFR gene, a series of reporter plasmid constructs was made using pBS1, pBS2, and the backbone of the pGL3-Basic reporter vector (Promega). Two subclones were obtained: pER318 and pER317, a 317-bp BamHI/SalI DNA fragment (−318 to −2) from pBS1 inserted at the HindIII vector site after ligation with the HindIII linker; pER1102 contained a 1101-bp fragment (−1102 to −2) and was obtained from the rat EGFR promoter fragment that was cut from pBS2 with NheI and then fused into pER317, which was also digested with NheI. More than 12 serial constructs using pER318 and pER1102 as a starting template with different deletions were obtained for promoter activity analysis. Deletions were made using unique restriction endonuclease sites. All constructs were verified by sequencing. Plasmid DNAs were prepared from these constructs using Maxi-prep kits (Qiagen) and quantitated by UV spectroscopy. A second plasmid pRL-TK (Promega), containing the Renilla luciferase gene under control of the thymidine kinase promoter, was prepared in a similar way and used as an internal control.

Chimeric pRL-TK-Luc reporter plasmid was constructed by cloning a HSV-TK promoter fragment from pRL-TK into the BglII and HindIII sites of pGL3-Basic. Heterologous promoter constructs of the rat EGFR promoter region (−318 to −260) were prepared by cloning different annealed oligonucleotides containing deletion or mutant TCC repeat sequences into pRL-TK-Luc at the NheI and XhoI sites. Plasmid DNAs from the clones were purified, and the presence of mutations within the −318 to −260 element was confirmed by sequencing.

Reporter Gene Assay—PC12 cells cultured on collagen-coated six-well plates (Nunc, Naperville, IL) were transfected using LipofectAMINE with 1 μg of the pER plasmid and 0.1 μg of the internal control pRL-TK (Promega), which contains Renilla luciferase downstream of the HSV-TK promoter. After NGF treatment (100 ng/ml) for 5 days, cells were transfected for 3 h. 9 h after transfection, cell lysates were prepared with the Dual-Luciferase Reporter Assay system (Promega), and both firefly and Renilla luciferase activities were measured in an LB 9507 luminometer (Berthold, Wildbad, Germany). The transfection efficiency was normalized according to the Renilla luciferase activity. The data are expressed as the means ± S.D. For rat EGFR promoter basal activity analysis, C6 and CV-1 cells were also transfected in the same way.

Nuclear Extracts and Electrophoretic Mobility Shift Assays—Nuclear protein extracts from PC12 cells were prepared according to Katagiri et al. (20). A duplex probe corresponding to the rat EGFR promoter sequence −318 to −260 was end labeled using [γ-³²P]ATP, T4 kinase, and forward reaction buffer (Life Technologies, Inc.) followed by incubation for 25 min at 37 °C. Binding reaction mixtures, which were preincubated at room temperature for 10 min, contained 10 μg of crude nuclear extracts in 10 μl Tris-HCl buffer, pH 7.5, 50 mM NaCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, 4% glycerol, 1 mM MgCl₂, and 2 μg of poly(dI-dC) (Amersham Pharmacia Biotech) and, for competition experiments, unlabeled competitor oligonucleotide (at 50-fold excess). Different annealed oligonucleotides containing deletion or mutant TCC repeat sequences and oligonucleotide sequences for Sp1 and AP2 response element-binding protein were used in the competition assays. Labeled probes (about 25,000 cpm) were added to the mixtures to a final volume of 15 μl and incubated for another 20 min at room temperature. The DNA-protein complexes and unbound probes were separated by electrophoresis through 4% polyacrylamide gels and detected by autoradiography.
RESULTS

Isolation and Characterization of the Rat EGFR Promoter and 5'-Flanking Region—To explore the down-regulation of the rat EGFR by NGF in rat PC12 cells, the promoter region of the rat EGFR gene was isolated from a rat P1 library. One P1 clone encoding the rat EGFR gene was obtained using PCR screening. Restriction fragments derived from the P1 clone GS17714 were subcloned into plasmids (Fig. 1).

Determination of the Transcription Start Site—Primer extension was employed to determine the transcription start site of the rat EGFR gene. Six products were seen from −268 to −78 relative to the ATG translation start codon (Fig. 2A). These products were observed in poly(A)⁺ RNA from both PC12 and rat C6 glioma cells. These data indicate that transcription of the rat EGFR gene is initiated in this region, and the 5'-most start site is located at −268 relative to the translation start site.

To map the region of the rat EGFR transcription start sites more precisely, 5'-RACE was performed using 5'-RACE-Ready rat kidney cDNA and a rat EGFR gene-specific primer. Major bands of about 1.4–1.6 kbp were obtained after amplification (Fig. 2B(i), lane 4). Southern blots of the amplified products were hybridized using a rat EGFR-specific internal probe that recognizes a sequence upstream of the 5'-RACE primer (Fig. 2B(ii)). These studies indicated that several rat EGFR-related sequences had been amplified. The products of the 5'-RACE PCR reactions, which resolved into multiple bands of approximately 1.4–1.6 kbp by Southern blot analysis, were subcloned and sequenced. From a total of 30 clones that were obtained form rat kidney cDNA, 24 were found which initiated from the same sites identified by primer extension. At least two RACE clones were identified for each of the start sites mapped by primer extension. Of the remaining six clones, PCR products that initiated from intermediate sites between the proximal and distal transcription start sites were observed. These clones may represent incompletely reverse transcribed sequences and could not be examined by primer extension. A summary of rat EGFR transcript start sites and a comparison with the corre-

![Image](http://www.jbc.org/figure/2)
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Cloning and Sequence Analysis of the 5'-Region of the Rat EGFR Gene

To determine the features of the promoter region responsible for transcriptional regulation, PC12 cells were transiently transfected with the rat EGFR promoter sequence (-1102 to -2 bp) and with promoter constructs with selective deletions, fused upstream of the luciferase reporter gene. The promoter activities were then determined by analysis of luciferase expression in the transfected cell extracts. The promoter activities were further analyzed by determination of the basal transcriptional control, a series of deletion constructs of the promoter region, comprising a promoter construct with a deletion extending to 5'-flanking region of the rat EGFR gene shows that a repeated sequence (TCCTCCTCC) was found at bp 88 and 89 of the coding region. The exon-intron boundary is shown in Fig. 3. The exon-intron boundary mapped in the rat receptor gene agrees with that reported for the gene for the human EGFR (21).

The nucleotide sequence upstream of the rat EGFR start sites contains neither a "TATA box" nor a "CAAT box" (Fig. 2C). Studies of transcription in vitro have shown that the TATA box serves to fix the site at which transcription will start (22). The nucleotide sequence further upstream of the ATG codon agrees with that reported for the gene for the human EGFR (21). In the sequence between nucleotides -540 and -1, which contains the putative promoter and the 5'-untranslated region, there is a G+C content of 64%; the region further upstream of -540 has a G+C content of only about 45%. Further analysis of the 5'-flanking region of the rat EGFR gene shows that a repeated sequence (TCCTCCTCC) was found at -343, -317, and -298. Similar sequences have been found in the upstream promoter region of the gene for human EGFR (Fig. 2C) and for chicken and mouse alpha2(I) collagen and appear to occur at sites sensitive to nuclease S1 (23, 24).

**Promoter Activity of the 5'-Upstream Region of the Rat EGFR Gene**

To determine the features of the promoter region responsible for transcriptional regulation, PC12 cells were transiently transfected with the rat EGFR promoter sequence (-1102 to -2 bp), and with promoter constructs with selective deletions, fused upstream of the luciferase reporter gene. The promoter activities were then determined by analysis of luciferase expression in the transfected cell extracts. The promoter activity was normalized with the Renilla activity in the same cell extracts. Activities of the EGFR promoter deletion constructs are shown in Fig. 4. To determine if other cell types exhibited a similar pattern upon DNA transfection, rat C6 glioma cells, known to express high levels of EGFR, and African green monkey kidney cells (CV-1), which express very low levels of EGFR, were also used (25, 26).

**Functional Analysis of the Rat EGFR Using Sequential Deletions**

To locate the region or regions essential for transcriptional control, a series of deletion constructs of the promoter region were prepared and used to transfect PC12, C6, and CV-1 cells. Comparing 5'-deleted constructs (Fig. 4A), it can be seen that 60% of maximum luciferase expression (pRE318) was retained when the deletion extended to -318. Further deletions to -260 and -181 (pRE260, pRE181) led to drastic reductions in luciferase expression. The results show that the 5'-most region, -1102 to -318, could be removed without substantially altering promoter activity. Thus, the nucleotide sequence between -318 and -2 is essential for basal transcription. Consistent with this, it was observed from the constructs with 3'-deletions (Fig. 4B) that there is no significant decrease upon deletion of -181 to -2 (pRE1102D181). Indeed, the lysates of the cells transfected with pRE1102D260 showed a 50% increase in luciferase expression compared with pRE1102. Further deletion to -318 (pRE1102D318) caused significant decreases in luciferase expression. Thus the major control of the basal transcription of the rat EGFR gene is between -318 and -260, and the first transcription start site -268 is just in this region.

**Functional Analysis of the Rat EGFR Promoter Using Internal Deletions**

To define the regulatory regions further, internal deletions were performed (Fig. 4C). pER318D260 contained only the sequences from -318 to -260. This 59-bp DNA fragment was expressed at a level that was 25% of the original pRE1102 activity. Two constructs lacking this region (pER1102D318-260, pER1102D318-181) exhibited markedly reduced promoter activity. These data indicate that the region from -318 to -260 and the transcription start site -268 may
FIG. 4. Activity of rat EGFR promoter constructs in PC12, C6, and CV-1 cells. Successive and internal deletions of the rat EGFR 5'-region were ligated to the luciferase reporter gene. The 3'-terminus of each deletion construct is nucleotide -2 relative to the rat EGFR translation initiation codon. 1 μg of each reporter gene plasmid and 0.05 μg of the internal control pRL-TK were transfected into PC12, C6, and CV-1 cells. 3 h after transfection, the solution was removed, and culture medium was added. 9 h after transfection, the cells were harvested, and luciferase activity was measured. The normalized luciferase activities were evaluated as a percentage of the untreated control and are presented as the means ± S.D. of triplicate values. The values are the means of triplicate values. Panel A, 5'-sequential deletions; panel B, 3'-deletions; panel C, internal deletions.
FIG. 5. Rat EGFR promoter activity in NGF-treated PC12 cells. After 5 days in culture in the presence or absence of NGF, PC12 cells were transfected with 1 μg of each reporter gene plasmid and 0.05 μg of pRL-TK. After transfection the solution was removed, and culture medium was added. 9 h after transfection the cells were harvested, and luciferase activity was measured. NGF was present throughout for the NGF-treated cells. Open bars, untreated control cells; black bars, NGF-treated cells. The normalized promoter activity estimated by firefly luciferase activity and normalized to Renilla luciferase activity derived from pRL-TK. NGF differs from untreated control in each transfection with a p value of at least <0.05. Error bars indicate ± S.D. Each experimental point was done in triplicate.
be important for maximal promoter function. This result also correlates with the previous result in Fig. 4B; luciferase expression was reduced drastically when cells were transfected with pRE1102D318. However, as shown in Fig. 4C, luciferase expression was increased 100% when transfected with pER1102D260–181 or pRE318D260–181 compared with pRE1102 and pRE318, respectively. This suggests that this region (–260 to –181) may not be necessary for rat EGFR transcription. The reason for the increase is not clear, but it may indicate the presence of a negative regulatory element in this region.

Identification of the NGF-responsive Sequences in the Rat EGFR Promoter—Different rat EGFR constructs were used in naive PC12 and in cells treated with NGF in order to localize the sequences responsible for NGF-induced decrease in transcription. Inhibition of the rat EGFR promoter (48–74%) was observed in NGF-treated PC12 cells with naive cells with the several constructs (Fig. 5), although in some lower basal activity constructs such as pRE260, pRE181, pRE1102D318, and pRE1102D318–260, these numbers may not be completely reliable. Most interestingly, after NGF treatment, the normalized rat EGFR promoter activity of nonoverlapping pRE1102D318–181 and pRE318D260 transfectants was decreased 76% and 72%, respectively. These results suggest the presence of at least two and possibly more NGF-responsive sequences in this construct (pRE1102) which are necessary for NGF inhibition.

Analysis of the Binding Activities to the NGF-responsive Elements by Gel Mobility Shift Assays—To demonstrate a nuclear protein factor(s) specific for binding to the 59-bp region extending from –318 to –260 in the rat EGFR promoter, nuclear protein-DNA interaction was detected by reduced electrophoretic mobility on a polyacrylamide gel. As shown in Fig. 6, A and B, two major complexes (A and B) were formed with nuclear extracts from untreated PC12 cells, but complex A was decreased with nuclear extracts from NGF-treated PC12 cells. The specificity of these complexes for the sequence was shown by a competition experiment in which the signals from both complexes were abolished completely by competition with a 50-fold excess of unlabeled probe (Fig. 6A, lanes 2 and 6; Fig. 6B, lane 3). In contrast, the same molar excess of a DNA fragment containing an Sp1 site failed to compete complex A (Fig. 6A, lanes 3 and 7; Fig. 6B, lane 4), and a DNA fragment containing an AP2 site failed to compete either of the complexes (Fig. 6A, lanes 4 and 8).

The specificity of TCC repeat sequences and Sp1 motif for complexes A and B was examined further in the competition assays with unlabeled probe and oligonucleotides that contain the wild type and mutant TCC repeat and Sp1 sequences (Fig. 6B). The unlabeled Sp1 motif itself competed effectively for complex B (Fig. 6B, lane 5), whereas the oligonucleotide containing the mutant Sp1 binding consensus failed to compete (Fig. 6B, lane 7). The unlabeled and single site mutant TCC repeat sequences competed effectively for complex A (Fig. 6B, lanes 6 and 9–15), whereas the oligonucleotide containing the multiple sites mutant TCC repeat sequences failed to compete (Fig. 6B, lane 8). These results indicate that the 59-bp region contains NGF-responsive elements for transcription factor binding, which are specific for TCC repeat sequences, but neither Sp1 nor AP2.

The TCC Repeat Sequences Appeared to Be Responsible for the Down-regulation of the EGFR by NGF—To determine whether TCC repeat sequences are functionally responsible for the down-regulation of the EGFR by NGF, we cloned oligonucleotides containing the 59-bp duplex fragments extending from –318 to –260 bearing wild type, deletion, or mutant elements (for Sp1 and TCC motif) upstream of the HSV-TK-LUC promoter reporter plasmid and transfected into untreated and NGF-treated PC12 cells (Fig. 7). Inhibition of the rat EGFR promoter (67–78%) was observed in NGF-treated PC12 cells compared with control cells with the constructs containing TCC repeat sequences and single site mutant TCC repeat sequence.
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DISCUSSION

The rat EGFR gene promoter has been isolated from cloned genomic DNA from a rat P1 library. The rat EGFR gene, like the human EGFR gene, does not contain typical TATA or CAAT boxes, and, also like the human gene, initiation of RNA transcription occurs at multiple sites. The 5'-flanking region of this gene is G+C-rich (64%), has one Sp1 binding site, and three repeats of the sequence TCCTCCTCC. The overall homology of rat EGFR gene promoter with that of human EGFR is approximately 62%.

The function of the 5'-promoter sequences in the transcription of the receptor has been examined here by the construction of deletion mutants. The variation in DNA uptake between individual DNA preparations and separate transfection experiments was normalized by quantitating the amount of genomic DNA from a rat P1 library. The rat EGFR gene promoter was retained by a 317-bp (-region of the rat EGFR gene, does not contain typical TATA or CAAT C-rich class found in a number of genes. In contrast to TATA-containing promoters, which accurately initiate at a single site, only a few of these G+C-rich promoters initiate transcription from a single site (29, 30), whereas many demonstrate multiple transcription start sites (31–33). G+C-rich sequences play a critical role in controlling the expression of various genes, including housekeeping genes and many cellular oncogenes.

The differentiation of PC12 cells by NGF involves striking morphological and biological changes including the induction or repression of numerous proteins required for the acquisition of a differentiated phenotype similar to that of a sympathetic neuron (34). One of the proteins decreased during this differentiation is the EGFR. It is possible that this down-regulation is part of the mechanism by which NGF instructs PC12 cells to stop dividing, because EGF is a mild mitogen for these cells (14). Although it is clear that the down-regulation is transcriptional, the detailed mechanism of this down-regulation is not known. Because there are clearly many genes whose transcription is decreased during such differentiation, it is possible that the EGFR can serve as a model with which to understand the mechanism of the down-regulation of key proteins by NGF in PC12 cells.

By using a series of deletion and mutation reporter constructs, we have shown that a 59-bp region on the rat EGFR promoter is transcriptionally activated in untreated PC12 cells and is transcriptionally inhibited in NGF-treated PC12 cells. There are at least two sites in this region which may mediate the basal promoter activity and NGF action: the TCCTCCTCC motif and the Sp1 binding site. By electrophoretic mobility shift experiments with a single copy of a rat EGFR promoter sequence, bp –318 to –260 was shown to bind nuclear proteins

FIG. 7. The TCC repeat sequences appeared to be responsible for the down-regulation of the EGFR by NGF. The 59-bp duplex fragments extending from –318 to –260 bearing wild type, deletion, or mutant elements (for Sp1 and TCC motif) were cloned in front of the HSV-TK luciferase promoter reporter plasmid and transfected into untreated and NGF-treated PC12 cells as described in the Fig. 5 legend. The sequences of the promoter fragments are shown at the left with the mutated sites underlined.

The best defined eukaryotic RNA polymerase II promoters contain proximal TATA elements that control the transcription start site and distal sequences that bind proteins that regulate transcriptional activity (27). Two types of promoters lacking TATA elements have been identified. In one type, an element called the initiator dictates accurate basal transcription from a start site within its sequence (28). The second type of TATA-less promoter is the G+C-rich class found in a number of genes. The function of the 5'-promoter sequences in the transcription of the receptor has been examined here by the construction of deletion mutants. The variation in DNA uptake between individual DNA preparations and separate transfection experiments was normalized by quantitating the amount of Renilla luciferase DNA taken up by the cells in each transfection. Approximately 60% activity of the 1-kbp 5'-region of the rat EGFR gene promoter was retained by a 317-bp (-318 to -2) fragment. Removal of an additional 58-bp (-318 to -260) region reduced the activity 2-fold, indicating the presence of positive control elements within this region. The sequence from -318 to -260 alone also showed a substantial basal level of activity after transfection and transient assay. However, removal of the other 80 bp (-260 to -181) increased the activity 2-fold, indicating the possibility that negative regulatory elements are located in this region. In the human EGFR gene, the sequence -140 to +80 also serves as a negative element (21). In addition, two TCCTCCTCC motifs and one Sp1-binding element are located between -318 and -260. Primer extension and 5'-RACE analysis indicate that the first and the major transcription initiation site is also located in this region.
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from both untreated and NGF-treated PC12 cells. One of the two complexes that was found to bind specifically to the TCC repeat sequences was decreased after NGF treatment. No difference was found between NGF-treated and untreated PC12 cells using Sp1 consensus oligonucleotides as probe. By reporter assays, the inhibition was abolished when using the constructs containing only Sp1 binding motif, no TCC repeat sequences or completely mutated TCC repeat sequences, respectively. These results indicate that down-regulation of rat EGFR is not caused by Sp1 binding but by altered binding of proteins to the TCCTCCTCC repeat sequences. TCC repeat sequences are required for the down-regulation of the rat EGFR by NGF in PC12 cells. The proteins binding to the TCC repeat sequences might be activators, and decreased binding of these unknown proteins may provide a mechanism to diminish rat EGFR promoter activity by preventing its activation. Further experiments are needed to define precisely the transcription factors binding to these sites.

In this study, we have found that the TCC repeat sequences of the promoter region appear responsible, at least in part, for the down-regulation of the EGFR by NGF. The more detailed mechanisms responsible for down-regulation of the rat EGFR by NGF in PC12 cells remain to be described. In this regard, experiments are under way to characterize the protein factors binding to TCC repeat sequences.

Acknowledgment—We thank Dr. Philip Lazarovici for advice on P1 cloning service.

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doi: 10.1074/jbc.275.10.7280

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