A Constitutive Promoter Directs Expression of the Nerve Growth Factor Receptor Gene

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Expression of nerve growth factor receptor is normally restricted to cells derived from the neural crest in a developmentally regulated manner. We analyzed promoter sequences for the human nerve growth factor receptor gene and found that the receptor promoter resembles others which are associated with constitutively expressed genes that have housekeeping and growth-related functions. Unlike these other genes, the initiation of transcription occurred at one major site rather than at multiple sites. The constitutive nature of the nerve growth factor receptor promoter may account for the ability of this gene to be transcribed in a diverse number of heterologous cells after gene transfer. The intron-exon structure of the receptor gene indicated that structural features are precisely divided into discrete domains.

Survival and maintenance of sympathetic and sensory neurons and cholinergic neurons of the basal forebrain are crucially dependent upon the action of nerve growth factor (NGF) (10, 25, 48). An essential step in NGF action is binding to a specific receptor which is expressed on the surface of cells derived from the neural crest. NGF bound to receptors is taken up by neurons and retrogradely transported to their cell bodies (21, 23), where vital cellular processes such as neurotransmitter enzyme synthesis and neuronal survival are directly influenced. Regulation of the effects of NGF on the development of the nervous system is dictated, in part, by the expression of the genes for both NGF and the NGF receptor molecules.

NGF is produced in low levels by a wide variety of target organs innervated by sympathetic and sensory neurons. Protein and mRNA measurements of NGF in peripheral organs have shown that the amount of NGF produced can be roughly correlated with the density of innervation (22, 40, 41). The pattern of expression for NGF indicates there is no strict tissue specificity. Analysis of the mouse NGF gene has revealed at least four different mRNA transcripts that are derived by alternative splicing and different promoter utilization (8, 39).

The receptor for NGF has been detected on neuronal cells and neuroblastoma tumors and also on non-neuronal derivatives of the neural crest such as melanomas (9), PC12 cells (13, 17), Schwann cells (46), and neurofibromas (36). Characterization of the receptor in rat sympathetic nerve and PC12 (47) cells and in human melanoma cells (14) reveals a glycosylated transmembrane protein that is phosphorylated predominantly on serine residues. Equilibrium binding of 125I-labeled NGF to cells reveals two distinct affinity states (24, 38, 45). The biochemical basis for these two affinity sites is not known.

The gene encoding the human NGF receptor was efficiently transfected into mouse fibroblasts by cotransformation with total genomic DNA and the herpesvirus thymidine kinase gene (4). Human NGF receptor sequences were rescued from a transfected mouse L-cell line by using human Alu repetitive sequences. Genomic clones were isolated that were capable of expressing the gene in fibroblasts. A single mRNA species of 3.8 kilobases (kb) was detected in melanoma and neuroblastoma cells as well as in rat superior cervical and dorsal root neurons and pheochromocytoma cells (3, 31).

By using genomic fragments as probes, NGF receptor cDNAs were isolated from a library made from the human melanoma cell line A875 (20). The full-length cDNA predicts a 28-amino-acid signal sequence, an extracellular domain containing four 40-amino-acid repeats with six cysteine residues at conserved positions followed by a serine/threonine-rich region, a single transmembrane domain, and a 155-amino-acid cytoplasmic domain. A large 3'-untranslated region exists in the receptor mRNA. The sequence of a full-length PC12 NGF receptor clone (31) indicates that the rat NGF receptor has considerable homology (>90%) with the human melanoma receptor.

The NGF receptor is differentially expressed and regulated during the development of the nervous system (3, 15, 32, 54). Whereas an increase in mRNA levels was detected in sympathetic ganglia in early development, a decrease in steady-state levels of receptor mRNA in sensory ganglia was observed over the same period (3). The differential, tissue-specific expression of the NGF receptor and the efficient transcription of the gene in fibroblasts led us to investigate what sequence elements promote transcription of this gene. Here we describe the structure of the human NGF receptor gene and the promoter structure of the gene that may play a role in its regulation.

MATERIALS AND METHODS

Cell culture and transfection. Mouse L cells were maintained in Dulbecco modified Eagle medium with 10% calf serum (GIBCO Laboratories). Human A875 melanoma cells were grown in Dulbecco modified Eagle medium plus 10% fetal calf serum, and PC12 cells were grown in Dulbecco modified Eagle medium plus 5% horse serum and 10% fetal calf serum. Cells were transformed with 2 µg of chloramphenicol acetyltransferase (CAT) plasmid DNA and 15 µg of L-cell DNA. After 48 h, the CAT assay was carried out.

Screening of genomic libraries. Escherichia coli strains K-802 and C-600 were used for plating the human genomic Charon 4A library (27) and the human placenta genomic library (33), respectively. In each case replica filters were
screened by using standard procedures (26). Hybridization was performed overnight in 50% formamide–1× Denhardt solution–5× SSCPE. After an initial wash at room temperature with 2× SSCPE, the filters were washed to 0.5× SSCPE (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C and autoradiographed for 1 to 2 days at −70°C.

**Nucleic acid blotting and hybridization.** Nitrocellulose filters containing transferred RNA or DNA (43) were baked in vacuo at 80°C, prehybridized in 50% formamide–5× SSCPE–5× Denhardt solution–500 μg of denatured salmon sperm DNA per ml at 42°C for at least 4 h, and then hybridized overnight at 42°C in 50% formamide–5× SSCPE–1× Denhardt solution–100 μg of denatured salmon sperm DNA per ml–10% dextran sulfate–denatured probe. Nick translation of restriction fragments was carried out as described previously (26) to specific activities of 5 × 10⁸ cpm/μg. Filters were washed to 0.4× SSC at 68°C and autoradiographed at −70°C with an intensifying screen.

**Oligomer mapping.** Partial digestion and restriction site mapping on the bacteriophage clones were analyzed by the method of Rackwitz et al. (30). The gel was blotted onto DE-81 cellulose paper for about 2 h, after which the paper was air dried and autoradiographed with Kodak X-OMat film for several hours at room temperature.

**S1 nuclease analysis.** Mapping by S1 nuclease digestion was carried out by the method of Weaver and Weissman (52). RNA was isolated as done by Chirgwin et al. (5). Total RNA (50 μg) was mixed with 5'-end-labeled (with T4 polynucleotide kinase and [γ-³²P]ATP) or 3'-end-labeled (with Klenow polymerase and α-²⁸P-deoxynucleoside triphosphates) probe in 10 μl of hybridization buffer containing 80% formamide–0.04 M PIPES [piperazine-N,N'-bis(2-ethanesulfonic acid)] (pH 6.5)–0.001 M EDTA–0.4 M NaCl. The reaction mix was heated for 10 min at 70°C and then allowed to anneal at 52°C for 12 h. After hybridization, 300 μl of buffer containing 0.03 M NaOAc, 0.25 M NaCl, 2 mM ZnSO₄, 20 μg of salmon sperm DNA per ml, and 300 U of S1 nuclease (Boehringer Mannheim Biochemicals) per ml was added immediately, and the mixture was incubated at 37°C for 30 min. Carrier RNA (20 μg) was added, and the samples were ethanol precipitated, electrophoresed on 6% polyacrylamide–8 M urea gels, and autoradiographed overnight at −70°C.

**Primer extension.** Synthetic oligomers were labeled with [γ-³²P]ATP and T4 polynucleotide kinase to specific activities of 10⁹ cpm/μg and used in primer extension experiments as described by Treisman et al. (49). The labeled oligomer (10 ng) was precipitated with 50 μg of total cellular RNA and then suspended in 10 μl of 80% formamide–1 mM EDTA–0.4 mM NaCl-0.04 M PIPES (pH 6.5) and allowed to hybridize overnight. Following hybridization, samples were ethanol precipitated and suspended in 10 μl of 0.1 M NaCl–1 mM EDTA–10 mM Tris hydrochloride (pH 8.3). To each sample was added 15 μl of reverse transcriptase buffer (10 mM dithiothreitol, 16 mM MgCl₂, 1 mM concentrations of each deoxynucleoside triphosphate, 750 U of RNase inhibitor per ml, 100 μg of actinomycin D per ml, 100 mM Tris hydrochloride, pH 8.3) and 15 U of reverse transcriptase. After 1 h at 42°C, reaction products were ethanol precipitated and subjected to electrophoresis on 6% polyacrylamide–8 M urea gels.

**DNA sequencing.** Genomic fragments were isolated by glass elution from agarose gels (51), subcloned into M13mp18 or M13mp19, and sequenced by the dyeoxyde chain termination technique (37). Oligonucleotide primers synthesized from receptor cDNA sequence were also utilized to sequence splice junctions, which were located at a significant distance from the restriction sites used for subcloning.

**CAT assay.** Genomic inserts were subcloned in plasmid pG3CATF, which contains a polylinker sequence upstream of the CAT gene (44). For the promoter-CAT construction, a 1.2-kb AvaII fragment was isolated, treated with DNA polymerase Klenow enzyme, and subcloned in the Sma site of pG3CATF in both orientations. The AvaII fragment begins at a site in the untranslated region of the first exon.

After transfection of these plasmids, CAT assays were performed as described by Gorman et al. (11). Cells were scraped off, dispersed by vortexing in 100 μl 0.25 M Tris (pH 7.8), and then disrupted by three freeze-thaw cycles, each consisting of an ethanol-dry ice incubation (5 min) followed by 37°C incubation (5 min). The debris was spun down, and extract was assayed for CAT activity by mixing 20 μl of extract, 5 μl of [¹⁴C]chloramphenicol (40 to 50 mCi/mmol), 35 μl of water, 70 μl of 1 M Tris (pH 7.8), and 20 μl of 4 mM acetyl coenzyme A. The reaction mix was incubated for 30 min at 37°C and then extracted with 0.6 ml of ethyl acetate. The ethyl acetate was dried in a vacuum aspirator, suspended in 30 μl of fresh ethyl acetate, and spotted onto a silica gel thin-layer plate. Chromatography was carried out with chloroform-methanol (95:5 ascending). The plate was exposed to X-ray film overnight at −70°C.

**RESULTS**

**Genomic map of the human NGF receptor gene.** A total of nine overlapping bacteriophage clones that span the entire NGF receptor gene were isolated. The initial clone for the receptor gene, LR1, was isolated from a mouse fibroblast cell line that expressed the human gene after DNA-mediated gene transfer (4). Subsequently, additional genomic clones were identified using receptor cDNA sequences and also genomic fragments as probes to screen bacteriophage libraries. Phages LR1, LR3, AR9, and λR11 (Fig. 1) were isolated from a partial Alul/HaeIII Charon 4A library, using a 5-kb BamHI genomic fragment that contained receptor sequences. Phages λ8C1, λ7C2, and λ10B1 were identified in a human placenta genomic library, using a 500-base-pair (bp) AvaI/EcoRI restriction fragment from the 5' end of the full-length receptor cDNA. Two additional overlapping phage clones, λ6 and λ7A2, were isolated from the placenta DNA library, using a 0.8-kb BamHI genomic fragment from the 5' region of 10B1. The λ6 and λ7A2 phage clones also hybridized to a 150-bp restriction fragment generated from the most 5' cDNA sequence. Consequently, λ6 and λ7A2 bacteriophage clones contain the most 5' receptor sequences (see below).

Inserts from the nine phage clones encompass a total of 47 kb. Hybridization of full-length and partial cDNA clones (20) together with DNA sequencing of cloned segments reveal that the human NGF receptor gene extends 23 kb and is divided into six exons. Table 1 displays the exon-intron junctions for the human NGF receptor. No deviations from the consensus splice site sequences (29) were found in the 5' donor and 3' acceptor splice sites.

The 5'-untranslated and signal peptide sequences are contained in the first exon. The second and third exons encode the N-terminal amino acid sequence of the mature receptor protein and a discrete domain of 161 amino acids, for which 24 are cysteine residues. This region can be divided into four repeats of 40 amino acids, with each repeat containing six cysteines in conserved positions (20). It is of interest that the splice site for the third exon is located...
TABLE 1. Intron-exon boundaries of the human NGF receptor gene*  

<table>
<thead>
<tr>
<th>Exon boundary</th>
<th>DNA sequence</th>
</tr>
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<tbody>
<tr>
<td>Signal peptide</td>
<td>GACGGGCCGCGCCTGCTGCTGCTGCTGCTTCTGGGG gtaggagccg D G P R L L L L L G</td>
</tr>
<tr>
<td>N terminus</td>
<td>tcctctatccag GTGCTCTTGGAGCGCTGGAGCGCTTCTGGGG gtaggtaggg V S L G C L D</td>
</tr>
<tr>
<td>Cysteine rich</td>
<td>tccgtctccag GCGTGACGTCCTGCGAGCGCTTGCTCTGGGG gtaggtgccgt S V T P E C E</td>
</tr>
<tr>
<td>Transmembrane</td>
<td>agctctctccag AGATCCCTGGGACGCTGGAGCGCTTGCTCTGGGG gtagagaggg W N S G Q</td>
</tr>
<tr>
<td>Cytoplasmic</td>
<td>ctctgcttcag GTGGAACGCTGCTGGAGCGCTTGCTCTGGGG gtagagccgg E I P G A F K R</td>
</tr>
<tr>
<td>COOH terminal</td>
<td>ctgtttcttcag CCCTCAAGGGTGACGAGCGCTTGCTCTGGGG A L K G D G G L Y S S L P</td>
</tr>
</tbody>
</table>

* The DNA sequence at the 5′ and 3′ boundaries of the six exons of the receptor gene are listed. Intron sequences are in lowercase letters, and exons are in capital letters. Nucleotide sequences were determined by dideoxy sequencing of M13 clones containing genomic fragments.

precisely after the 24th cysteine residue. Similarly, the transmembrane region found in the fourth exon contains an intron-exon boundary in the stop transfer sequence directly following the stretch of hydrophobic amino acids (Table 1). Such precise divisions of the structural features of the receptor molecule are supportive of a functional role of these domains.

The mRNA for the NGF receptor contains a large 3′-untranslated region. This sequence is uninterrupted and is contained in the largest receptor exon (2,291 bp), together with sequences representing the intracellular domain of the receptor. In addition, a small exon contains a portion of the putative cytoplasmic region of the receptor.

**Analysis of the 5′ end of the NGF receptor gene.** The 5′ end of the receptor gene was localized to a 4-kb BamHI restriction fragment from λ6 by specific hybridization with a 150-bp BglII/EcoRI restriction fragment, representing the extreme 5′ end of the full-length receptor cDNA. In addition, a 45-base oligonucleotide complementary to nucleotides −11 to −55 (see Fig. 4) hybridized specifically to this fragment. To

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**FIG. 1.** Map of overlapping genomic clones containing NGF receptor sequences. The gene is shown in a 3′-to-5′ orientation, with exons represented as boxes at the bottom. Nine phage λ clones were mapped by conventional techniques including oligomer mapping and by Southern blot analysis with NGF receptor cDNA probes. Selected fragments were subcloned into pSP64 and pT3/T7 plasmids and characterized by DNA sequencing of exon-intron junctions (Table 1).
determine the 5' exon structure and the promoter region of the NGF receptor, the 4-kb BamHI fragment was subcloned. Further restriction mapping revealed that 5'-untranslated sequences were confined to a 0.5-kb Sma fragment within the 4-kb BamHI fragment (see Fig. 4).

To locate precisely the mRNA start sites, S1 nuclease protection was carried out. A 370-bp Ncol/Sma genomic fragment was end labeled at the Ncol site. When this labeled DNA probe was hybridized to total human melanoma RNA, digested with S1 nuclease, and subjected to electrophoresis on a denaturing gel, a major protected fragment of 152 nucleotides was observed, together with a minor fragment of 151 nucleotides (Fig. 2). The size of the protected fragments indicates that the major start points of transcription are eight and nine nucleotides beyond the 5' end of the published full-length cDNA sequence (20). Larger protected fragments of much lower intensity than the two major bands were also detected. These consist primarily of a cluster of fragments 160 to 180 nucleotides in length and a single 250-nucleotide-long fragment. These fragments most likely represent minor upstream start sites.

To verify the receptor transcription start site, the 45-base oligonucleotide to the 5'-untranslated region was used in primer extension experiments. This antisense oligonucleotide was 5'-end labeled and hybridized to RNA isolated from the human melanoma A875 cell line. A875 cells express abundant numbers of NGF receptors (9). After treatment with reverse transcriptase, the products of the primer extension reaction were displayed on a denaturing gel (Fig. 3). Two extension products of 99 and 100 nucleotides were observed, corresponding precisely to the positions deduced from the S1 analysis. No additional bands of larger molecular size were produced in the primer extension.

Primer extension was also carried out on RNA isolated from the rat pheochromocytoma PC12 cell line to determine whether the transcription initiation site used in the human melanoma cell line represents the universally used initiation site. Receptors expressed in the A875 cell line are predominantly of the low-affinity class. On the other hand, PC12 cells express both high- and low-affinity NGF receptors and respond to NGF by neuronal differentiation. To exclude the possibility of multiple 5' ends of the receptor gene in PC12 cells, an oligonucleotide primer complementary to nucleotides -48 to -76 of the rat cDNA sequence, where +1 denotes the A of the initiating methionine (31), was used to map the rat receptor initiation site. The extended product is 75 nucleotides in length (Fig. 3), in good agreement with the predicted start site for the human gene. Thus, the site of transcription initiation is the same for the receptor gene in rat PC12 and human melanoma A875 cells.

**Human NGF receptor promoter.** The sequence of the region upstream of the first coding exon is shown in Fig. 4. Exon I encodes the signal peptide of the immature receptor protein and is followed by a 6.5-kb intron. The sequence composition of this genomic region contains a high G+C content (73%) but neither a "TATA" nor a "CAAT" consensus sequence. As is the case with a number of eukaryotic promoters, there are four conserved GGCCGG sequences within 200 nucleotides of the initiator ATG codon. These GC-rich sequences represent potential binding sites for transcription factor Sp1 (6, 7).

The predominant mRNA start sites are at nucleotides -121 and -122. The Sp1 core binding sites (GGCCGG) are upstream of these initiating nucleotides, at positions -164, -176, -197, and -303. Sp1 binding sites were first detected in the 21-bp repeats of the simian virus 40 early promoter within 80 nucleotides of the initiation site. Three of the four putative Sp1 binding sites in the receptor promoter are within 80 bases of the major initiation site.

The ability of this upstream sequence to support transcription was tested with the bacterial CAT gene in a transient expression assay (11). A 1.2-kb AvaII restriction fragment (-29 to -1,200) was cloned on the 5' side of the CAT gene in pG3CATF (44) in both orientations. The ability of these constructs to express CAT activity was assayed by transfe-
We have previously observed stable expression of human NGF receptors after gene transfer of human genomic DNA and several phage clones into mouse fibroblasts (4). Expression of receptors was detected by immunological screening of transfected cells, using monoclonal antibodies against the human receptor (36), and also by binding of $^{125}$I-labeled NGF followed by affinity cross-linking. The ability of the receptor promoter to transcribe the CAT gene in transient assays of fibroblasts is a partial explanation for why this tissue-specific gene is expressed so efficiently after gene transfer in fibroblasts.

**DISCUSSION**

The human NGF receptor gene is a single-copy gene, consisting of six exons that span 23 kb. The entire gene is capable of being transferred to fibroblast cells from human genomic DNA and expressed at high levels. As many as 500,000 receptors per cell can be detected after gene transfer (4). A single mRNA species is detected in nerve cells and in cultured cells expressing both high- and low-affinity receptors. The two kinetic forms appear to be encoded by the same protein (12); however, the molecular basis of the two forms is unresolved.

We have determined the 5' end of the receptor mRNA by using both primer extension and S1 mapping. The sequence of the receptor promoter is extremely GC rich. Consensus transcription sequences such as TATA and CAAT are notably absent. A number of eucaryotic genes that have promoters high in G+C content lack the characteristic TATA and CAAT boxes. These include the hypoxanthine guanine phosphoribosyltransferase gene (28), the 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase gene (34), the 3-phosphoglycerate kinase gene (42), the adenosine deaminase gene (50), and the adenine phosphoribosyltransferase gene (2), all of which encode enzymes with housekeeping functions. Expression of these genes is at a low constitutive level in a wide number of different tissues. In addition, the promoters of several cellular growth control genes such as c-Ha-ras (18), c-Kirsten ras (16), and the EGF receptor (19) also do not contain the typical TATA and CAAT transcriptional consensus sequence, but have multiple GC box motifs.

Hence, the promoter for the NGF receptor resembles those genes which are constitutively expressed or involved in growth regulation. A common structural feature among this class of promoters is multiple GGGCGG sequences in the 5' upstream region. These sequences are similar to the GC boxes in the 21-bp repeat region of the simian virus 40 promoter and are recognized by transcription factor Sp1 (7). Since all of these gene products are in some way involved in growth control and their promoter elements are similar, the regulation of their expression may involve the binding of related transcription factors.

An unusual feature of this promoter is that it appears to direct transcription primarily from two adjacent nucleotides instead of using multiple sites of initiation. Primer extension of PC12 RNA similarly did not reveal multiple start sites. Other potential upstream initiation sites are detected in the S1 analysis of human mRNA, but these are extremely weak. Most constitutively expressed genes contain multiple initiation sites distributed over a 15- to 20-base region that are all used with the same frequency (35). For the receptor promoter, specificity of transcription initiation must be controlled by regulatory elements other than the consensus TATA sequence.
NGF RECEPTOR GENE STRUCTURE

FIG. 4. Nucleotide sequence of the 5' region of the human NGF receptor gene. (A) Detailed map of the 5' end of the NGF receptor gene. The coding sequence (exon I) is shown by the solid box along with relevant restriction sites. (B) Nucleotide sequence of the 5' region of the human NGF receptor. The sequence of the first exon is shown together with the predicted amino acid sequence. Nucleotide residue +1 denotes the A of the ATG used to initiate translation of the receptor protein. The initiation sites determined by primer extension and S1 nuclease protection experiments are denoted by arrows. The repeated sequence GGGCGG is enclosed in a box. Within the first exon, an oligonucleotide of 45 nucleotides (underlined) was synthesized for primer extension experiments.

In the course of characterizing the genomic organization of the receptor gene, we noted that the phage inserts used in transfection studies (4) lacked the 5' promoter region and also sequences representing the first two exons (Fig. 1). Phage DNAs (λR1, λR5, λR9, and λR11) were cotransfected into mouse fibroblasts in pairwise combinations, using the herpesvirus thymidine kinase gene (tk) as a selectable marker (4). We observed positive transfected colonies expressing the human NGF receptor at a rate of 1 in 500 to 1,000 tk+ colonies. The lines were identified and subsequently purified by repeated rounds of rosetting. Three cloned cell lines displayed NGF receptors that bound 125I-labeled NGF specifically and were immunoprecipitated by monoclonal antibodies to the NGF receptor. In some lines transfected with λR9 phage alone, receptors were also detected on the cell surface. We are investigating the possibility that an internal promoter sequence within the receptor gene is responsible for this expression after gene transfer.

During neural crest migration, only a selective subpopulation of cells express NGF receptors and are responsive to NGF (1). The constitutive nature of the receptor promoter implies that selective activation of the receptor gene during development is dependent upon other regulatory elements. On the other hand, reports of transient expression of NGF receptors in mesodermal tissues during early development (32) may reflect unregulated use of the receptor promoter.

FIG. 5. Transient expression of the CAT gene directed by the NGF receptor promoter. Mouse fibroblast L cells were transfected with 30 μg of plasmid DNA and 20 μg of high-molecular-weight carrier DNA per 100-mm dish containing 5 × 106 cells. CAT assays were conducted 48 h later. An autoradiogram is shown of the following experimental conditions. Lane A. Cells transfected with pSV2CAT which contains the simian virus 40 early promoter 5' of the CAT gene; lane B. untransfected cells; lane C. cells transfected with pBat4 which contains the 1.2-kb Avall fragment in a 5'-to-3' orientation with the CAT gene (the 1.2-kb fragment extends 5' from an Avall site in the untranslated region of the first exon [Fig. 4]); lane D. cells transfected with pBat2, which represents the 3'-to-5' orientation of a 1.2-kb Avall fragment with the CAT gene.
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