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trans Activation of Nerve Growth Factor in Transgenic Mice Containing the Human T-Cell Lymphotropic Virus Type I *tax* Gene

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Three lines of transgenic mice containing the human T-cell lymphotropic virus type I (HTLV-I) *tax* gene develop neurofibromas composed of perineural fibroblasts (S. H. Hinrichs, M. Nerenberg, R. K. Reynolds, G. Khoury, and G. Jay, Science 237:1340–1343, 1987; M. Nerenberg, S. H. Hinrichs, R. K. Reynolds, G. Khoury, and G. Jay, Science 237:1324–1327, 1987). Tumors and tumor cell lines derived from these mice produce neurite outgrowth from PC-12 cells and nerve growth factor (NGF), as determined by RNA (Northern) blot analysis and enzyme-linked immunosorbent assays. In vitro cotransfection studies demonstrate that Tax is able to *trans* activate the NGF promoter in NIH 3T3 fibroblast cells. The major *cis*-acting *tax*-responsive element in the NGF promoter (AGGGTGTGACGA) has 92% homology with a *tax*-responsive element contained within the 21-bp repeats of the HTLV-I long terminal repeat. The receptor for NGF is also expressed in the transgenic tumor cells, suggesting that Tax may activate an autocrine mechanism through the upregulation of NGF.

Human T-cell leukemia virus type I (HTLV-I) is a retrovirus etiologically associated with adult T-cell leukemia (21, 32, 35, 50) as well as the neurodegenerative disorder HTLV-I-associated myelopathy, or tropical spastic paraparesis (3, 11, 23, 31, 36). The pathogenesis of these disorders remains unclear but is thought to involve the deregulation of endogenous cellular genes by the HTLV-I *trans*-acting protein Tax.

Activation of growth factors and receptors is thought to be an important mechanism of transformation (47) and may be involved in other disease processes. Previous studies have shown that Tax is able to *trans* activate the genes for interleukin-2 (IL-2) (27, 28, 43), a potent growth factor for T-cell proliferation; IL-2 receptor (22, 27, 43); the proto-oncogene c-*fos* (8); granulocyte-macrophage colony-stimulating factor (GM-CSF) (28, 29); and vimentin (25), as well as the HTLV-I long terminal repeat (LTR) (6, 9, 45). It has been postulated that Tax may stimulate T-cell proliferation, perhaps through activation of an autocrine mechanism of transformation via the IL-2/IL-2 receptor pathway, which may be important in the development of leukemia by HTLV-I (22, 27, 43).

Transgenic animals containing the HTLV-I *tax* gene develop neurofibromas composed of perineural fibroblasts (20, 30). In vivo *trans* activation of the cellular genes for the hematopoietic growth factor GM-CSF and the IL-2 receptor by Tax has been demonstrated in these transgenic animals (15). Activation of other growth factors by Tax might also occur in these transgenic animals and be involved in the development of their abnormal phenotype. Further understanding of such Tax-induced responses could lead to better understanding of mechanisms involved in HTLV-I-related diseases.

In addition to the development of neurofibromas, Tax expression in these transgenic animals is associated with proliferation of ductal epithelial cells of the salivary glands (16) and tumors in the iris and adrenal medulla (14), tissues which have been reported to produce nerve growth factor (NGF) (7, 19, 40, 42). Four potentially Tax-responsive *cis*-acting elements were found to be contained in the NGF promoter region by computer homology search. Because of these observations, investigations were undertaken to determine whether Tax is capable of influencing NGF production. These studies demonstate that Tax is able to transactivate the murine NGF promoter.

MATERIALS AND METHODS

Derivation of transgenic mice. Derivation of transgenic animals containing the HTLV-I long terminal repeat *tax* gene has previously been described (30). Three lines of HTLV-I *tax* transgenic mice have been maintained for several years.

Preparation of tissues and tumor cell lines from animals. Animals were killed, and dissected tissues were frozen at −70°C for subsequent RNA extraction. Fresh tissues were used for protein extraction. The establishment of tumor cell lines PX-1 and PX-3 has been described previously (15). Cell line CN-1 was derived in a similar manner from a cranial nerve tumor from transgenic founder series 6-2. Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS; GIBCO Laboratories, Grand Island, N.Y.), 100 U of penicillin per ml, and 100 μg of streptomycin per ml (Quality Biological, Inc., Gaithersburg, Md.).

Northern (RNA) blot analysis. RNA was extracted by the RNA_{zol} method (Cinna/Biotecx, Friendswood, Tex.). RNAs were separated by electrophoresis through 1% agarose–formaldehyde gels, transferred to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, N.H.), and hybridized to ^{32}P-labeled cDNA probes prepared by random oligolabeling (Pharmacia, Piscataway, N.J.). The probe for NGF was kindly provided by W. Rutter (41), and the probe for NGF receptor (NGFR) was kindly provided by E. Shooter (34). The *tax* probe has previously been described (30).

NGF ELISA analysis. Sandwich enzyme-linked immunosorbent assays (ELISAs) were performed on tissue extracts

and tissue culture-conditioned medium with anti-mouse β (2.5S)-NGF monoclonal antibody and anti-mouse β (2.5S)-NGF-β-galactosidase monoclonal antibody (Boehringer Mannheim, Indianapolis, Ind.). Protein extraction from tissues and ELISAs were performed as described before (Boehringer Mannheim protocol). Control standard solutions of NGF were prepared from purified 7S NGF (U.S. Biochemical Corp., Cleveland, Ohio).

Bioassay for neurite outgrowth. The rat pheochromocytoma PC-12 cell line was used in a functional assay for factors capable of producing neurite outgrowth, particularly NGF, as originally described by Greene and Tischler (18). Unconditioned and conditioned media were diluted between 1:2 and 1:10 and added to PC-12 cells grown in DMEM with 5% FCS and 10% horse serum (GIBCO Laboratories). Cells were examined after 48 h to determine whether dendritic outgrowth had occurred. Unconditioned and conditioned media were also preincubated with sheep anti-mouse NGF (kindly provided by G. Guroff) to block the NGF effect on PC-12 dendritic outgrowth.

Construction of plasmids. The wild-type (wt) NGF-Cat construct was made by replacing sequences of the human growth hormone (hGH) gene with the chloramphenicol acetyltransferase (CAT) gene in the NGF-hGH plasmid pSam7, kindly provided by G. Heinrich (51). The 1.5-kb *Bam*HI-*Bgl*II hGH fragment of pSam7 was replaced with the 0.8-kb *Bam*HI fragment of pCM-4 (Pharmacia) containing the CAT gene. dl-475 was derived from NGF-Cat by deleting the 5′ NGF promoter sequences to the *Eco*RV site at −475 bp; dl-252 was made by deleting the 5′ sequences to the *Eco*RI site at −252 bp; dl-154 was constructed by deleting the 5′ region to the *Bsp*MI site at −154 bp; and dl-0 was derived by excising the entire promoter region to the *Bam*HI site. The remaining 5′ promoter deletion constructs were created by polymerase chain reaction (PCR) amplification mutagenesis. Various primers at the 5′ end were designed to introduce *Hin*dIII sites at particular points in the NGF promoter; the same 3′ primer containing a region of the CAT gene, including the *Bam*HI site, was used for all PCR syntheses. The PCR-generated fragments were cloned into the *Hin*dIII-*Bam*HI fragment of pNGF-Cat following removal of the wt NGF fragment. Constructs were generated with 5′ deletions at −123, −83, −72, and −49 bp. The sequences of all constructs were confirmed by DNA sequencing (T_7 Sequenace; Pharmacia). HTLV-I-Δtax was made by deleting the *tax*-containing *Bgl*II fragment of HTLV-I-PX (30).

Transfection of cells and CAT assays. A total of 1.5×10^6 NIH 3T3 cells (grown in DMEM plus 10% FCS, 100 U of penicillin per ml, and 100 μg of streptomycin per ml) were plated per 100-cm^2 tissue culture dish on the day prior to transfection. The medium was changed 3 h prior to transfection. Calcium phosphate transfection was performed as described previously (13). Then, 5.0 μg of a CAT-containing plasmid, 1.0 μg of the Tax-producing plasmid HTLV-I-PX or HTLV-I-Δtax (as a control plasmid), and 19 μg of pUC19 (used as carrier DNA) were transfected per dish. The medium was changed after overnight incubation. Cells were harvested at 48 h posttransfection, and CAT assays were performed as described previously (12).

Cross-linking of [^{125}I]NGF to the NGFR. [^{125}I]NGF-NGFR cross-linking was performed as described previously (17). Briefly, 2×10^6 cells were harvested in phosphate-buffered saline (PBS; GIBCO Laboratories) with 1 mM EDTA and washed three times with PBS. Cells were resuspended in PBS (pH 6.5), and 25 ng of [^{125}I]NGF (82.4 μCi/μg; New England Nuclear, Boston, Mass.) was added. After incubation at 37°C for 30 min, the cross-linking agent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl (Pierce Chemical, Rockford, Ill.) was added to a final concentration of 4 mM and incubated at room temperature for 30 min. Cells were washed twice with PBS and solubilized overnight on ice in 100 μl of PBS with 2% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1 mM *N*-tosyl-L-phenylalanine chloromethyl ketone (Boehringer Mannheim). The extract was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gels were dried, and autoradiography was performed. Competition studies were performed by preincubating samples for 30 min with a 200-fold molar excess of unlabeled NGF.

Computer sequence analysis. Computer homology searches were performed with the DNA Star program (DNA Star, Inc., Madison, Wis.).

RESULTS

NGF production by tumor cells and tumor cell lines. Conditioned medium from tumor cell lines PX-1, PX-3, and CN-1, derived from neurofibromas of *tax* transgenic mice, was shown to cause neurite outgrowth from rat pheochromocytoma-derived PC-12 cells (Fig. 1). Within 24 h of the addition of conditioned medium, the PC-12 cells became less rounded and flatter and began to extend dendritic processes, which became maximal at 48 h and persisted for at least 7 days. In control experiments, as little as 1 ng of purified NGF per ml was able to elicit minimal dendritic outgrowth from PC-12 cells, which could be maximized with the addition of 10 to 50 ng of purified NGF per ml.

Conditioned medium from the PX-3 and CN-1 cell lines was less effective on PC-12 dendritic outgrowth than PX-1-conditioned medium but was able to produce a clear response. Dendritic outgrowth stimulated by tumor cell-conditioned medium could be reduced by up to 50% by the addition of sheep anti-mouse NGF antibody. Since fibroblast growth factor (49) and interleukin-6 (38) have been shown to be capable of producing neurite outgrowth in PC-12 cells under certain conditions, ELISAs for NGF were performed. The higher-expressing Tax tumor cell line PX-1 also secreted more NGF than tumor cell lines PX-3 and CN-1 (PX-1, 8.8 ± 3.2 μg of NGF per ml; PX-3, 0.65 ± 0.33 ng/ml; CN-1, 1.0 ± 0.1 ng/ml).

Northern blot analysis was performed to confirm the presence of NGF and to correlate the expression of NGF to that of *tax* in tumors and tumor cell lines (Fig. 1E). A ^{32}P-labeled murine NGF cDNA probe (specific activity, $>1 \times 10^9$ cpm/μg of DNA) was hybridized to a Northern blot containing 10 μg of polyadenylated RNA per lane. No NGF mRNA was detectable in PC-12 cells (Fig. 1E, lane 1), although a minimal 1.2-kb message was detected in control tail tissue from a normal mouse (transgenic littermate). Tumor tissue and cell lines PX-1 and PX-3 produced significant levels of NGF mRNA. The same lanes were hybridized with a ^{32}P-labeled *tax* probe. The levels of NGF mRNA appeared to generally correlate with the levels of *tax* mRNA.

***tax trans* activates the NGF promoter.** The observation that *tax* expression seemed to correlate with NGF expression suggested that *tax* might *trans* activate the NGF promoter. To test this possibility, an NGF promoter-CAT construct was derived. When transfected into mouse fibroblast NIH 3T3 cells along with a non-*tax*-producing plasmid (pHTLV-I-Δtax), minimal CAT activity was observed (Fig. 2A). (The plasmid pHTLV-I-Δtax was used to control for possible effects on NGF promoter function by competition of tran-

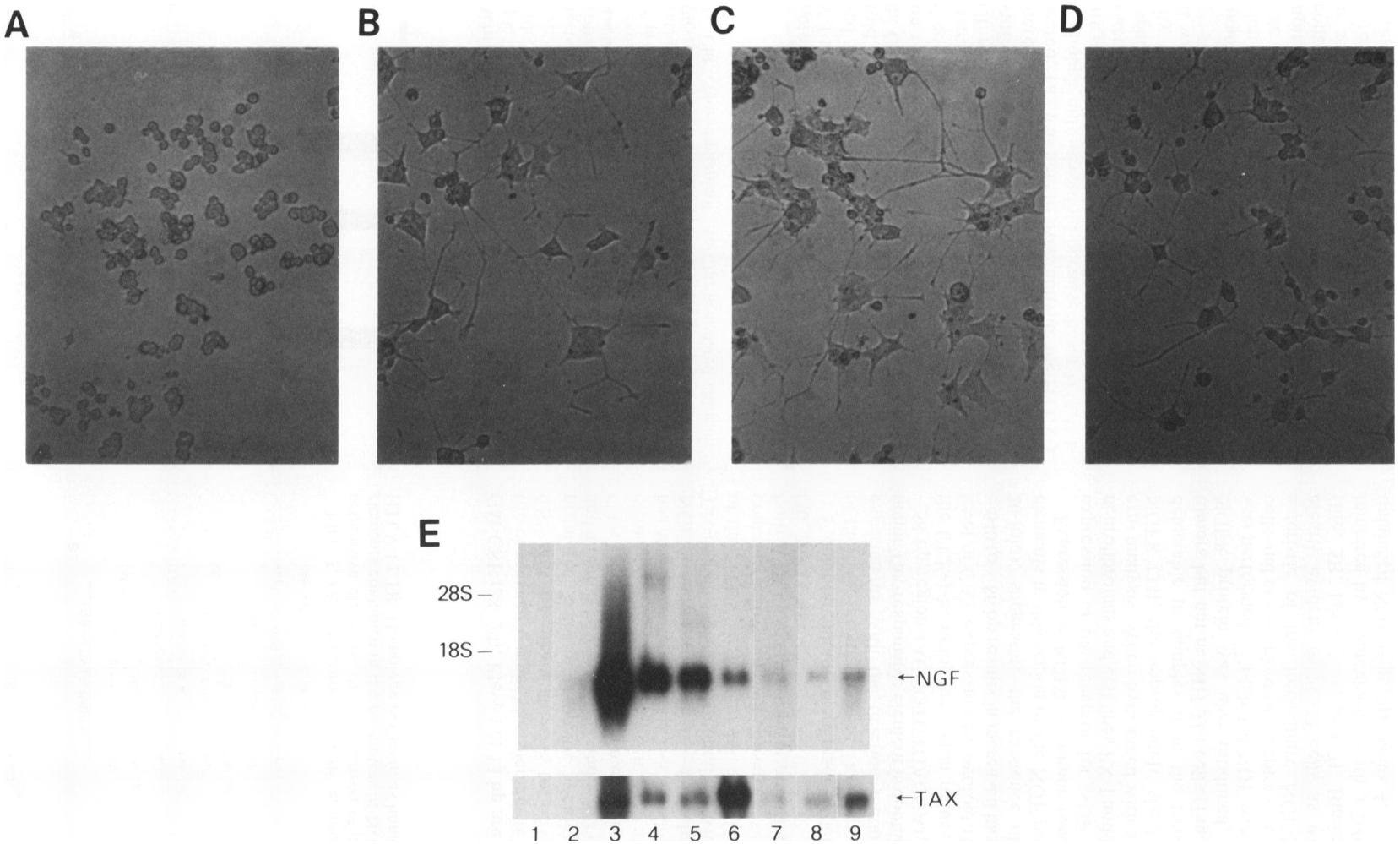

FIG. 1. Response of PC-12 cells after 5 days to (A) DMEM with 10% FCS, (B) PX-1-conditioned medium, (C) PX-3-conditioned medium, and (D) CN-1-conditioned medium. Conditioned medium was diluted 1:3. Magnification, ×140. (E) Northern blot analysis for NGF. Ten micrograms of poly(A) selected RNA was electrophoresed through a 1% agarose–formaldehyde gel and transferred to nitrocellulose; blots were probed with random oligo-^{32}P-labeled NGF cDNA and *tax* cDNA probes. Lane 1, PC-12 cells; lane 2, normal mouse tail; lane 3, PX-1; lane 4, PX-3; lane 5, salivary gland (founder line 6-2); lanes 6 through 9, peripheral tumors.

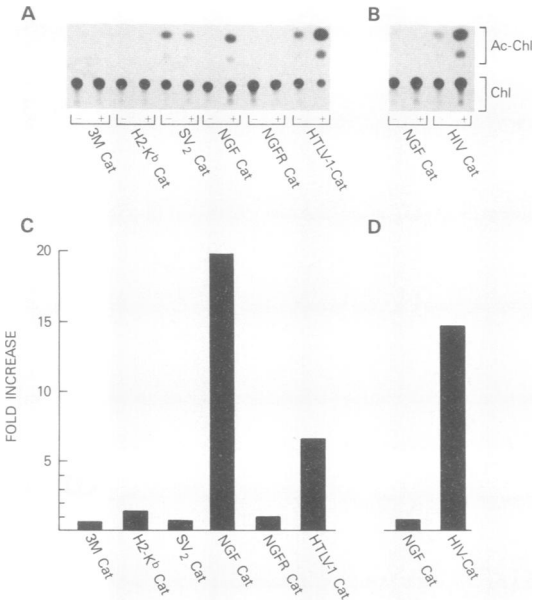

FIG. 2. Activation of NGF promoter by HTLV-I *tax* in cotransfection assays. (A) Representative CAT assays of promoter-CAT constructs with (+) and without (−) cotransfection of HTLV-I *tax*; (B) CAT assays of promoter-CAT constructs with (+) and without (−) cotransfection of HIV *tat* (24). (C and D) Increase in CAT activity by cotransfection with *tax* or *tat* as in panels A and B as measured by scintillation counting. Values represent the average of at least four experiments. 3MCat, no promoter; H2-K^b-Cat, murine class I *H2-K^b* promoter (4); SV_2Cat, simian virus 40 early promoter (12); NGF-Cat, promoter of murine NGF; NGFR-Cat, promoter of human NGFR (39); HTLV-I-Cat, HTLV-I LTR (46); HIV-CAT, HIV LTR (1). Chl, chloramphenicol; Ac-Chl, acetylated chloramphenicol.

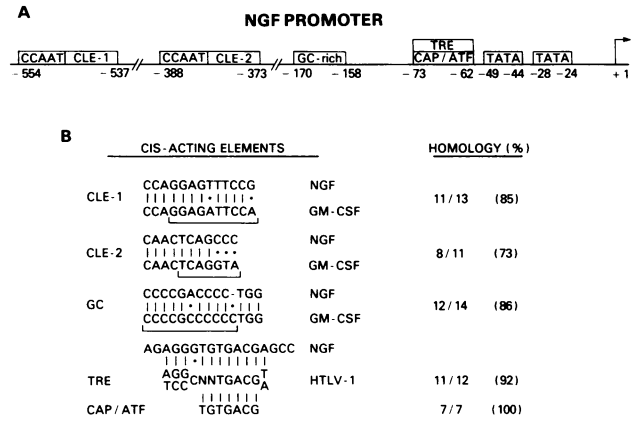

FIG. 3. (A) Structure of NGF promoter and potential Tax-responsive elements. (B) Comparison of potential Tax-responsive elements contained in the promoters of NGF and GM-CSF and the HTLV-I LTR. Homology is shown as homologous/total base pairs.

scription factors for regulatory sequences contained within the pHTLV-I-tax plasmid.) However, when NGF-CAT was cotransfected with the Tax-producing plasmid HTLV-I-PX (30), a 20-fold increase in CAT activity was observed (Fig. 2A and C). CAT activity correlated with levels of RNA transcription (data not shown). Cotransfection of *tax* with other CAT constructs, including pCAT3M (no promoter), pSV_2CAT (simian virus 40 early promoter) (13), and pBat4 (human NGF receptor promoter) (39), showed no significant *trans* activation effect in NIH 3T3 cells, while a slight (1.5-fold) increase was detectable with the *H2-K^b* promoter (4). As expected, Tax was able to *trans* activate the HTLV-I LTR (45), although only sevenfold because basal HTLV-I-CAT activity was relatively high.

tax trans activation of the NGF promoter was shown to be somewhat specific, as HIV-Tat (24) was unable to stimulate CAT activity when cotransfected with NGF-CAT. As expected, Tat was able to stimulate HIV-LTR-CAT (1) (Fig. 2B and D).

NGF promoter contains a Tax-responsive element. The cotransfection studies described above demonstrated that Tax was able to *trans* activate the 640-bp region 5′ of the NGF promoter. To determine whether this region of the promoter contained any potential Tax-responsive elements, a computer search to identify regions with homology to known Tax-responsive elements was performed. Four potential Tax-responsive elements were found (Fig. 3). The NGF promoter contains regions with significant homology to three Tax-responsive elements found in the promoter for GM-CSF (28, 29). Eleven of 13 bp are homologous between the conserved lymphokine element (CLE)-1 region of GM-CSF and the region at −548 to −537 of the mouse NGF promoter. Eight of 11 bp are homologous between CLE-2 and a region between −382 and −372 of the NGF promoter. CAAT boxes are found immediately adjacent 5′ to these elements. The region between −169 and −156 of NGF also has 86% homology with CLE-3. In addition, the region of the NGF promoter from −71 to −58 bp has 92% homology to a Tax-responsive element contained within the 21-bp repeats of the HTLV-I LTR (10, 48). This Tax-responsive element contains an overlapping consensus sequence responsive to both ATF (activating transcription factor) and cyclic AMP (10, 26, 33, 48).

5′ deletion mutants were made to determine which regions of the NGF promoter were Tax-responsive. Cotransfection experiments with the deletion mutants revealed that the major Tax-responsive element is contained within the −71 to −58 bp region (AGGGTGTGACGA), although deletion of the CLE-1-homologous region reduced the Tax-responsive level of CAT expression by twofold (Fig. 4). Deletion from −640 to −86 bp results in increased basal transcription (up to 20-fold), suggesting the existence of negative regulatory elements in this region of the NGF promoter (Fig. 4).

Expression of NGFR by tumor tissue and cell lines. The expression of the receptor for NGF was demonstrated in neurofibromas and cell lines PX-1 and PX-3 by RNA (Northern) analysis. When hybridized with a ^{32}P-labeled probe for NGFR (34), the appropriately sized 3.8-kb message was detectable in samples from the transgenic cell lines and tumors but not in NIH 3T3 cells (Fig. 5A). To confirm that NGFR protein was also produced, protein cross-linking was performed. ^{125}I-labeled NGF was incubated with tumor cells and cross-linked to receptors. A 103-kDa species characteristic of the low-affinity NGF-NGFR complex was clearly evident in PC-12 cells as well as in tumor cells (Fig. 5B, lanes 3, 5, and 7). Binding of [^{125}I]NGF was inhibited by preincubation with a 200-fold molar excess of unlabeled NGF (Fig. 5B, lanes 4, 6, and 8). As expected, NGFR was not detectable in NIH 3T3 cells (Fig. 5B, lanes 1 and 2).

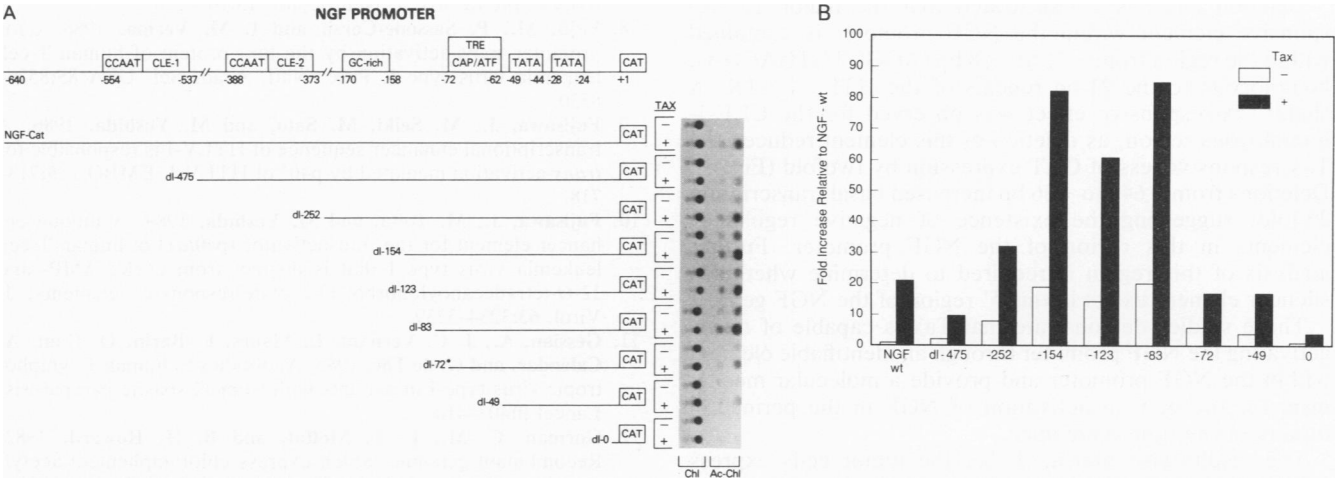

FIG. 4. Deletion analysis of NGF promoter. (A) Schematic diagram of potential Tax-responsive *cis*-acting elements in the NGF promoter. Progressive deletions of promoter regions are numbered according to the base pair at which 5′ deletion occurs. dl-72* contains point mutations within a Tax-responsive element (TRE) which has 92% homology with a TRE found in the 21-bp repeats of the HTLV-I LTR: HTLV-I TRE, (A/T)(G/C)(G/C)CNNTGACG(T/A); NGF wt, AGGgTGTGACGA; dl-72*, gGCCAGTGcCaA. Transfections and CAT assays were performed as described in the legend to Fig. 2. (B) Increase in CAT activity of deletion mutants relative to uninduced NGF wt CAT activity.

DISCUSSION

The mechanism of HTLV-I-induced diseases remains poorly understood but is thought to involve the dysregulation of cellular genes important in the regulation of cell growth. The *trans*-activating protein Tax is able to increase transcription from the HTLV-I LTR (6, 9, 45), as well as the genes for IL-2 (27, 28, 43), IL-2 receptor (22, 27, 43), c-*fos* (8), GM-CSF (28, 29), and vimentin (25). Activation of GM-CSF and IL-2 receptor had been demonstrated in earlier studies of these lines of transgenic mice carrying the HTLV-I *tax* gene (15). The *tax*-induced overproduction of GM-CSF in the transgenic mice correlated with significant physiological changes, including polymorphonuclear leukocyte infiltration into the GM-CSF-producing neurofibromas, granulocytosis, and myelomonocytic hyperplasia of the spleen and bone marrow (15).

Further characterization of these transgenic mice indicated that in addition to the formation of perineural fibromas (20, 30) and salivary ductal cell proliferation (16), tumors of the adrenal medulla, iris, and ciliary bodies also occur (14). Since these tissues have been reported to produce NGF in normal animals (7, 19, 40, 42), investigations were undertaken to determine whether there might be an association between *tax* and NGF expression.

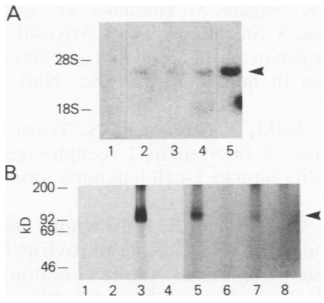

FIG. 5. Expression of NGFR by tumors and tumor cell lines. (A) Northern blot analysis for NGFR. Lane 1, mouse fibroblast NIH 3T3 cells; lane 2, tumor cell line PX-1; lane 3, tumor cell line PX-3; lane 4, tumor from transgenic founder series 6-2; lane 5, PC-12 cell line. Arrow indicates 3.8-kb NGFR message. (B) Cross-linking of [^{125}I]NGF to NGFR. Lane 1, NIH 3T3 cells; lane 3, PC-12 cells; lane 5, tumor cell line PX-1; lane 7, tumor cell line PX-3. Lanes 2, 4, 6, and 8, same as lanes 1, 3, 5, and 7, respectively, except cells were preincubated with a 200-fold molar excess of unlabeled NGF. Arrow indicates 103-kDa [^{125}I]NGF-NGFR complex.

Tumors and tumor cell lines from the *tax* transgenic mice were shown to produce NGF, as determined by ELISA and RNA analysis (Fig. 1D). Conditioned media from the tumor cell lines stimulated neurite outgrowth from PC-12 cells (Fig. 1). However, since antibody to NGF could not completely abolish this effect on PC-12 cells, other factors produced by the tumor cell lines could also contribute to the PC-12 dendritic outgrowth. Non-NGF neuronal growth factors have been reported in patients with neurofibromatosis (44). The levels of NGF production in the tumor cells appeared to correlate with the levels of Tax production. Although this suggested a Tax effect on NGF, a molecular mechanism was sought to understand this potential Tax activation of NGF.

Preliminary cotransfection studies demonstrated that Tax was able to transactivate the 640-bp 5′ promoter region for NGF (Fig. 2), although HIV-Tat, a transactivating protein from another human retrovirus associated with neurological disease, was unable to *trans* activate NGF. This suggested that the ability of Tax to transactivate the NGF promoter was not a general property of retroviral *trans*-acting proteins but had some specificity for Tax.

Analysis of the NGF promoter revealed that four potential Tax-responsive elements were present (Fig. 3). Three of the elements had striking homology to elements within the GM-CSF promoter which have been shown to be Tax-responsive, including CLE-1 and CLE-2 as well as a G+C-rich region (28, 29). The fourth potential Tax-responsive element was found to have 92% homology with a Tax-responsive element contained within the 21-bp repeats of the HTLV-I LTR (10, 33, 48). This Tax-responsive element contains an overlapping consensus sequence previously shown to be responsive to both ATF (activating transcription factor) and cyclic AMP (26, 33).

Deletion analysis demonstrated that the major Tax-responsive element within the NGF promoter is contained within the region from −71 to −58 bp (AGGGTGTGACGA), homologous to the 21-bp repeats of the HTLV-I LTR. A slight Tax-responsive effect was observed for the CLE-1-homologous region, as deletion of this element reduced the Tax-responsiveness of CAT expression by twofold (Fig. 4). Deletions from −640 to −86 bp increased basal transcription 20-fold, suggesting the existence of negative regulatory elements in this region of the NGF promoter. Further analysis of this region is required to determine whether a silencer element exists in this 5′ region of the NGF gene.

These studies demonstrate that Tax is capable of *trans*-activating the NGF promoter through an identifiable element within the NGF promoter and provide a molecular mechanism for the in vivo activation of NGF in the perineural tumors of the transgenic mice.

The results also indicated that the tumor cells express NGFR (Fig. 5). Perineural cells have previously been shown to express NGFR (2). Cotransfection studies were unable to demonstrate that Tax could *trans* activate the promoter for the human NGFR (Fig. 2A and C). It therefore seems likely that the tumor cells express a low, constitutive level of NGFR whose level of transcription is not directly influenced by Tax. The observation that Tax is capable of activating the murine NGF promoter in a cell which expresses the NGFR suggests that Tax might stimulate an autocrine mechanism in these perineural fibroblasts.

This is the first demonstration that HTLV-I *tax* is capable of altering the regulation of a gene involved in nervous system function. It remains to be determined whether HTLV-I infection influences NGF production in patients with HTLV-I-associated myelopathy and whether this contributes to the pathogenesis of this disorder.

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