Mutations in the parkin gene are common in early-onset and familial Parkinson’s disease (PD), and the parkin protein interacts in the ubiquitin-proteasome system as an E3 ligase. However, the regulatory pathways that govern parkin expression are unknown. In this study, we showed that a phylogenetically conserved N-myc binding site in the bi-directional parkin promoter interacted with myc-family transcription factors in reporter assays, and N-myc bound to the parkin promoter in chromatin immunoprecipitation assays and repressed transcription activity. Parkin expression was inversely correlated with N-myc levels in the developing mouse and human brain, in human neuroblastoma cell lines with various levels of n-myc amplification, and in an inducible N-myc cell line. Although parkin and N-myc expression were dramatically altered upon retinoic acid-induced differentiation of a human neuroblastoma cell line, modulation of parkin expression did not significantly affect either rates of cellular proliferation or levels of cyclin E. Analysis of additional genes associated with familial PD revealed a shared basis of transcription regulation mediated by N-myc and the cell cycle. Our results, in combination with functional knowledge of the proteins encoded by these genes, suggest a common pathway linking together PD, the ubiquitin-proteasome system, and cell cycle control.

Several genes have been identified that, when mutated, give rise to rare familial forms of Parkinson’s disease (PD). Elucidation of the functions of the proteins encoded by these genes is likely to enhance understanding of the mechanisms underlying the more common sporadic form of the disease. The most common identified cause of familial PD is mutation of the parkin gene. Parkin protein functions in the ubiquitin proteasome system (UPS) as an E3-ligase capable of catalyzing the transfer of ubiquitin to target substrates. Extensive efforts to identify parkin substrates have revealed numerous candidates, although a number of other studies have observed neuroprotective effects of parkin overexpression in cells simultaneously challenged with various insults (reviewed by Feany and Pallanck). Given the plethora of potential parkin substrates and numerous cellular pathways parkin might interact in, the timing and localization of parkin expression may be a critical feature of gene function.

Parkin expression in the mammalian brain is largely neuron-specific and developmentally regulated, perhaps implying a role for parkin in neuronal maturation. Evidence suggesting that the parkin gene is frequently mutated in breast and ovarian cancer is noteworthy in this regard, because it raises the possibility that parkin may be intimately involved in cell cycle regulation, perhaps even functioning as an effective tumor suppressor in the periphery. Parkin protein may directly interact in the cell cycle as a component of a Skp1-cullin-F-box-like ubiquitin ligase complex capable of targeting cyclin E for degradation. Disruption of cell cycle regulation could therefore be plausibly invoked as a mechanism leading to neuronal degeneration as a result of parkin dysfunction.

To explore the nature of parkin’s developmentally regulated pattern of expression and potential interaction in the cell cycle, we analyzed the parkin promoter for regulatory elements known to interact with transcription factors involved in cell cycle control. We identified a transcription regulation domain, conserved across several species, containing a sequence similar to an E-box motif, a promoter element known for interacting with the myc family of proteins. We demonstrate that parkin expression inversely correlates with expression of N-myc, a transcription factor critically involved in both neuronal development and tumorigenesis. N-myc also regulates transcription of other familial PD genes in an inducible cell line, thereby suggesting a common biological pathway among genes associated with PD.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Plasmids—** SK-N-BE (2)-M17, SH-SY5Y, SK-N-F1, IMR-32, and SK-N-DZ cells were cultured in Opti-MEM media (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. For differentiation of SK-N-NE (2)-M17 cells, serum content was reduced to 2% and retinoic acid (Sigma-Aldrich) was included at a concentration of 1 µM. The N-myc-inducible cell line (also known as Tet-21/N or SHEP-21/N cells) was provided by Dr. J. Shohet (Baylor College of Medicine, Houston, TX) with permission from Dr. M. Schwab (Deutsches Krebsforschungszentrum, Heidelberg, Germany). These cells were maintained in RPMI 1640 media (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin, grown with or without doxycycline (dox; 1 µg/ml). The parkin-inducible PC-12 cell line was provided by Drs. F. Davies and A. Bruce (Institut National de la Santé et de la Recherche Médicale, Paris, France), and maintained in Dulbecco’s modified Eagle’s medium media (Invitrogen) supplemented with 10% horse serum, 5% newborn calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin, 75 µg/ml geneticin, and hygromycin (50 µg/ml).
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ml), grown with or without doxycycline (1 µg/ml). Plasmids encoding the N-myc and max proteins in a mammalian expression vector were obtained from Dr. H. Kondo (Osaka University, Osaka, Japan). The vector pGL3-154, containing a portion of the parkin promoter, was described previously (12).

**Dual-luciferase Assay**—Cells were plated 24 h before transfection into 24-well culture plates at ~80% confluence, and transfection was performed with Fugene 6 reagent (Roche Biochemicals), using 0.2 µg of DNA per well in a 1:3 ratio of DNA/Fugene reagent, and added to cells in serum-free media for 12 h. Luciferase-containing constructs (pGL3) were co-transfected with pRL-SV40 renilla vector (Promega, Madison, WI) to control for transfection efficiency, in a molar ratio of 1:40 (pRL-SV40 versus pGL3 vectors). Forty hours after transfection, cells were rinsed with phosphate-buffered saline and then harvested with passive lysis buffer (Promega). The dual-luciferase system (Promega) was used according to the manufacturer’s protocol, and experiments were repeated in six wells. Readings were taken in duplicate on a Turner Designs 20/20 single injector luminometer (Promega).

**Chromatin Immunoprecipitation**—The N-myc-inducible cell line was plated at 10^5 cells per 10-cm dish and cultured for 5 days in the presence or absence of 1 µg/ml doxycycline. Cultures were rinsed with phosphate-buffered saline and cross-linked for 15 min at 37 °C in serum-free medium containing 1% formaldehyde. Media was then removed, and un-cross-linked formaldehyde was quenched by incubation in 125 mM glycine for 10 min at room temperature. Cultures were washed, and cells were collected by scraping into phosphate-buffered saline containing protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride and 1 mM EDTA, 1 mM pepstatin, benzamidine, and leupeptin). Cell pellets were re-suspended in 1 ml of lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris·HCl, pH 8.0) containing protease inhibitors and incubated on ice for 10 min. DNA was then sheared by sonication to an average size of 300 bp and samples were centrifuged. 0.2-ml supernatants from control and doxycycline-treated samples were diluted 1:10 with 0.1% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, and 16.7 mM Tris·HCl containing protease inhibitors, and 5% was set aside as input DNA. Samples were pre-cleared for immunoprecipitation by incubation with protein A-agarose blocked with 1 µg/ml of sonicated salmon sperm DNA, 1 mg/ml BSA, 1 mM EDTA, 10 mM Tris·HCl, pH 8.0. 5 µg of either rabbit anti-N-myc (Santa Cruz Biotechnology, Santa Cruz, CA) or control IgG were added to each sample, and incubations were continued overnight at 4 °C. Immune complexes were collected with Protein A-agarose and with 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris·HCl, pH 8.0, containing 500 mM NaCl, and 0.25 mM LCl, 1% Nonidet P-40, 1% deoxycholate, 10 mM EDTA, 10 mM Tris·HCl, pH 8.0, and finally Triton-EDTA. To simultaneously elute immune complexes and reverse cross-links, agarose-bound adducts were incubated at 65 °C in 1% SDS, 0.1 mM NaHCO3, 0.1 mM NaCl, and 0.2 mM NaCl. Samples of Input DNA were made 0.3 mM in NaCl and also incubated overnight at 65 °C. DNA was purified using the Qiagen PCR cleanup kit (Qiagen, Valencia, CA).

Real-time quantitative PCR analysis of the number of copies of parkin promoter DNA in input DNA, N-myc, or control IgG samples was performed on a Light Cycler system using Qiagen QuantITect SYBR-Green PCR reagents and primers that flank the parkin promoter E-box sequence (forward, 5'-TAG AAC TAC GAC TCC CAG CAG GC-3'; reverse, 5'-CGG CTC TCC TGG GTT AAA TCC TC-3'). For each sample analyzing the parkin E-box sequence, copy number of parkin promoter DNA was determined by comparison to a standard curve composed of 10-fold serially diluted plasmid DNA containing parkin promoter sequence versus the crossing-over point. Control primers amplifying 4 kb upstream of the parkin E-box sequence were also designed (forward, 5'-ACC TGT CAG CCT TCC TTG AAA CTC TCC-3'; reverse, 5'-CCC AGA AAC AGC AAT CCT CAC TCC-3'). For each sample analyzing the parkin E-box sequence, copy number of parkin promoter DNA was determined by comparison to a standard curve composed of 10-fold serially diluted plasmid DNA containing parkin promoter sequence versus the crossing-over point. Control primers amplifying 4 kb upstream of the parkin E-box sequence were also designed (forward, 5'-AGG ATG AGG GAG CAC GAC GCC-3'; reverse, 5'-TAG AGG ATG AGG GAG CAC GCC-3').

**RESULTS**

**Identification of an Evolutionarily Conserved Potential N-myc Binding Sequence within the Parkin Promoter**—We identified previously an E-box motif in the parkin/PACRG promoter 46 bp upstream of the parkin transcription initiation site that interacted with nuclear protein derived from human substantive nigra and overlapped with a region responsible for transcription regulation (12). To identify functional domains near the parkin E-box motif, we sequenced the parkin promoter in a number of evolutionarily divergent species using PCR primers placed in the more highly conserved exonic regions of the parkin and PACRG genes. As a whole, the parkin promoter displayed a conservation of 52% between human and mouse, in contrast to 83% conservation in the parkin open reading frame. We resolved a palindromic phyloprint (evolutionarily conserved promoter element) containing the E-box motif (CCGGTTGCCG) 44 bp upstream of the parkin transcription initiation site (Fig. 1A).

Using the MatInspector protein binding prediction program (www.genomatix.de), a number of proteins from the myc family of transcription factors were predicted to interact with the parkin promoter E-box, with highest similarity to the N-myc consensus binding domain matrix (Fig. 1B). The CGCGTG motif in the Pax-3 promoter has previously been shown to bind both N-myc and max in gel-shift assays (14). The CGCGTG motif found in the parkin and pax-3 promoter differs from the canonical E-box sequence of CACGGTG by 1 base pair, yet both sequences are known to strongly interact with myc-family proteins (15). In humans and mice, neither the CGCGTG nor CACGGTG sequence motif is repeated within 20 kb of genomic sequence about the parkin promoter E-box.

To determine the importance of the parkin promoter E-box and myc protein expression on transcription regulation, a DNA construct containing sequence from the first exon of parkin to
the E-box sequence was inserted upstream of a promoter-less luciferase reporter gene in the pGL3-basic vector (pGL3-154). This plasmid was transiently transfected along with the control vector pRL-SV40 into the SK-N-BE (2)-M17 cell line, and strong transcription activation relative to control plasmid (pGL3-basic) was observed (Fig. 1 C), as we have reported previously (12). To examine whether N-myc might affect this transcription activity, reporter assays were conducted in parallel with the overexpression of the N-myc transcription factor, max, or both N-myc and max. In all cases, the transcription activity of the parkin promoter construct was reduced; however, transcription activity of the SV40 promoter (pGL3-SV40) was not significantly affected, thereby suggesting activity in pRL-SV40, used to control for transfection efficiency, was also unaffected. Taken together, these data suggest that N-myc repressed transcription activation in the parkin promoter reporter constructs.

Parkin Expression Is Down-regulated by N-myc—N-myc-induced down-regulation of transcription in reporter assays does not necessarily convey a physiologically significant interaction in which N-myc regulates parkin expression. To determine whether modulation of N-myc expression alters endogenous parkin levels, we first examined parkin expression in an N-myc-inducible (tet-off) cell line. This cell line is of human SH-EP neuroblastoma origin and endogenously possesses a single copy of the N-myc gene, which expresses protein at very low levels (16). Promoter constructs containing either the promoterless pGL3-Basic or the parkin promoter (pGL3-154) were transiently co-transfected in addition to transcription factor(s) into BE (2)-M17 cells and harvested 40 h later. The molar ratios of vectors in each experiment were conserved by supplementing with no-insert plasmid DNA (pcDNA3.1) as necessary. Error bars represent 2×S.E. of at least three independent experiments. *, p < 0.001; **, p < 0.0001, with respect to the transcription activity of pGL3-154.

**Fig. 1. Organization of the parkin/PACRG promoter and E-box motif.** A, parkin and PACRG's first exon are represented by black bars, with arrows indicating the direction of transcription. The parkin promoter was sequenced in the listed species and aligned relative to the E-box-like motif, with conserved sequence outlined. B, electronic prediction of transcription factors interacting with the conserved E-box sequence, as determined in silico using MatInspector v2.2. The position and orientation of the predicted interaction is given by the black arrows, relative to indicated sequence. C, luciferase activity (normalized to renilla activity) is given, where 1 unit equals the activity of the control vector, pGL3-SV40, driven by the SV40 early promoter, as determined using the dual-luciferase assay. Plasmids encoding either the promoterless pGL3-Basic or the parkin promoter (pGL3-154) were transiently co-transfected in addition to transcription factor(s) into BE (2)-M17 cells and harvested 40 h later. The molar ratios of vectors in each experiment were conserved by supplementing with no-insert plasmid DNA (pcDNA3.1) as necessary. Error bars represent 2×S.E. of at least three independent experiments. *, p < 0.001; **, p < 0.0001, with respect to the transcription activity of pGL3-154.
flank the E-box sequence in the parkin promoter, and quantification of precipitated DNA was achieved using a SYBR green fluorescence PCR system. In cells grown in the presence of dox, insignificant amounts of the parkin promoter were precipitated by a polyclonal antibody specific to N-myc, relative to IgG controls (Fig. 3, B–D). On the other hand, in cells grown without dox (and therefore high levels of N-myc; see Fig. 2, B and C), ~18 times the copies of the parkin promoter were immunoprecipitated relative to cells grown with dox (Fig. 3C), thereby demonstrating that N-myc interacts with the chromatin complex at or near the E-box sequence in the parkin promoter.

To further validate this system, we analyzed a region 4 kb upstream of the parkin E-box sequence in the parkin promoter that does not contain sequence similar to an E-box, and would not be expected to immunoprecipitate with the anti-N-myc antibody. Insignificant amounts of DNA immunoprecipitated in cells grown with or without dox when normalized to IgG controls (Fig. 3D). We also analyzed a known N-myc binding site by designing primers that flank an E-box motif in the human telomerase gene. In agreement with a previous study (17), approximately seven times the amount of DNA precipitated from cells grown without dox versus IgG or cells grown with dox (Fig. 3D).

**Parkin Expression Inversely Correlates with N-myc during Development and in Human Neuroblastoma Cell Lines**—Northern analysis of the amount of parkin and N-myc mRNA in poly-A purified RNA derived from mouse embryos showed parkin expression to be restricted to the latest stages of embryonic development (day 17), whereas N-myc expression was highest during mitotic stages of neuron growth (day 11; Fig. 4A), a
finding consistent with previously published results (18, 19).
Likewise, quantitative RT-PCR analysis of fetal and adult human brain tissue showed that roughly double the parkin mRNA is present in the adult brain versus that of the fetal brain, measured relative to control mRNA (Fig. 4B).

In addition to the developing mammalian embryo, N-myc expression is also highly variable in human neuroblastoma. Neuroblastoma accompanied by amplification of the N-myc gene is associated with enhanced tumor aggressiveness and a poor clinical outcome (20). Most neuroblastoma derived cell lines have N-myc amplification; however, both the SH-SY5Y line and the SK-N-F1 line possess only a single copy of N-myc. Western blotting was used to measure relative parkin and N-myc expression in various neuroblastoma cell lines. Again, parkin expression inversely correlated with N-myc amplification (Fig. 4C).

Retinoic Acid Up-regulates Parkin Expression—Retinoic acid is known to potently down-regulate N-myc expression and to induce growth arrest and differentiation in neuroblastoma cells (21). SK-N-BE(2)-M17 cells cultured for 10 days in media containing retinoic acid developed a complex network of cell processes indicative of differentiated and post-mitotic neurons (Fig. 5, A and B). Levels of N-myc and parkin were analyzed at days 3 and 10 of exposure to retinoic acid. N-myc mRNA decreased ~20-fold at the day 10 (data not shown), whereas parkin mRNA doubled relative to control mRNA (Fig. 5C). Western blot analysis demonstrated an increase of parkin protein in differentiated cells relative to control proliferating cells and a corresponding decrease in N-myc and cyclin E protein (Fig. 5 D).

Parkin Overexpression Does Not Affect Cellular Proliferation or Levels of Cyclin E—To determine whether parkin may play a role in the cessation of cellular proliferation, growth rates were measured, over the course of 96 h, in PC-12 cells that express inducible human parkin (tet-off system). Although the removal of dox in the N-myc-inducible cell line (thereby increasing N-myc expression) dramatically increased cellular proliferation (Fig. 6A, ■ versus □), as has been previously reported (16), increasing parkin expression by removal of dox from the PC-12 cell media did not produce a significant change in cell growth rate (Fig. 6A, ▲ versus △). Cell growth rates were also measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide cell proliferation assay, and similar results were obtained (data not shown).

Parkin has been proposed to participate in the degradation of cyclin E, an important regulator of the cell cycle, via the ubiquitin proteasome system (9). To determine whether modulation of parkin expression might affect endogenous levels of cyclin E, protein levels were measured in lysates derived from the parkin-inducible cells grown with or without dox. Modulation of parkin expression had no significant effect on levels of endogenous cyclin E protein relative to β-actin protein in this cell line (Fig. 6B).

**DISCUSSION**

Soon after the parkin gene was identified, Northern analysis revealed a broad distribution of parkin expression in the brain (22), and parkin regulation was described as being akin to a class of genes with ubiquitous expression known as “cellular housekeepers.” Contrary to these original speculations, in-depth examination revealed a neuron-specific and developmentally regulated pattern of expression in the brain (5, 6). Given this background, and in light of the current heterogeneity of hypothesized parkin functions, further investigation into the regulation of parkin expression may divulge details of how loss of parkin function might cause PD and whether parkin might be involved in cancer. The parkin gene contains massive introns and spans a common fragile site (7), making it one of the largest genes in the genome. Yet despite a first intron >280 kbp in size, another expanded gene (PACRG) lies mere 204 bp
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**Developmental regulation of parkin expression.** The targets of the N-myc transcription factor have been of interest because the discovery of N-myc amplification in human cancer. Those targets up-regulated are probably involved in the initiation and continuation of cell growth, and those down-regulated might be involved in differentiation. Parkin is known to possess E3-ligase activity, where target proteins are polyubiquitinated and degraded via the proteasome. Numerous tumor suppressor genes with E3-ligase activity and protein domains homologous to parkin protein have also been described, including MDM2 (24) and BRCA1 (25). It is intriguing that the Park protein (Parkin-like ubiquitin ligase) might also function as a tumor suppressor gene by regulating p53 activity (26). Given parkin’s relationship with N-myc and the implication of parkin as a tumor suppressor, we hypothesized that parkin might be a key component in mediating the transition from cell growth to cessation and differentiation. We performed basic experiments to determine whether parkin might affect cellular proliferation rates or levels of cyclin E but found no such evidence. However, additional studies that more directly measure parkin’s effect on tumorigenesis now seem warranted.

Another gene down-regulated by N-myc with a similar expression profile to parkin, NDRG1 (N-myc down-regulated gene 1), is involved in growth arrest and cell differentiation and is mutated in hereditary motor and sensory neuropathy-Lom (27). The involvement of autosomal-recessive loss of function mutations in NDRG1 in human peripheral neuropathy may be all the more relevant to parkin-linked disease, in that loss-of-function parkin mutations have also been identified in human patients with peripheral and sensory neuropathy (28, 29). It is conceivable that the mechanisms of neurodegeneration resulting from mutations in the parkin or NDRG1 gene overlap, at least in the peripheral nervous system.

Given N-myc’s profound effect on the cell cycle, microarray studies and SAGE analysis report a strikingly small number of genes affected by N-myc expression: 114 genes up-regulated, most of which deal with ribosome biogenesis and protein synthesis, and 70 down-regulated genes (30). It is therefore of interest that several of the genes currently associated with PD are affected by N-myc expression; α-synuclein and UCH-L1 were previously shown to be up-regulated by N-myc in the inducible N-myc cell line (30). We have duplicated these original findings in this cell line using quantitative PCR (data not shown), in addition to demonstrating that parkin is down-regulated by N-myc. N-myc also seems to regulate select components of the UPS, including proteasome subunits and heat shock proteins, thereby exerting downstream control of protein degradation and further implicating the importance of the UPS on cell cycle control. Thus, a common basis of transcription regulation among the genes associated with PD may be suggesting a biological overlap in endogenous gene functions.

The parkin gene (park2) is located within the common fragile site Fra6E in the human genome, associated with early-onset recessive PD (31), originally isolated as a c-Myc interacting protein and can transform cells in coordination with the oncogenes c-myc and/or c-ras (32) in addition to functioning as a circulating tumor antigen in human breast cancer (33). Likewise, UCH-L1 (Park5) demonstrates abnormal expression in cancer that correlates with tumor severity (34) and may contribute to the
degradation of tumor suppressor Kip1 (35). Last of all, overexpression of α-synuclein (Park1) results in increased cellular proliferation rates and an increase in the number of cells in the S phase (36). Thus, a provocative link between PD, the UPS, cell cycle control is demonstrated on both a genetic and functional level.

Breach of cell cycle checkpoints is probably one of the primary mechanisms by which postmitotic neurons undergo death (37). Although this mechanism of cell death may have obvious implications in the developing nervous system and cancer, the link to a progressive neurodegenerative disorder is less clear. Because the UPS is a central component of cell cycle control (38), neurons that have an attenuated UPS because of genetic abnormalities, inherent cellular properties, and/or environmental insults might be unable to maintain control over the cell cycle and ultimately succumb to death from inappropriate re-entry. Functional studies that bridge together the genes associated with PD into a common pathway may reconcile disease pathogenesis and provide rational approaches to therapeutically intervention.

Acknowledgments—We are indebted to the investigators who generously shared their reagents and expertise to make this study possible. The N-myc-inducible cell line was provided by Dr. J. Shohet with permission from Dr. M. Schwab. The parkin-inducible cell line was provided by Drs. F. Darios and A. Brice. Plasmids encoding the N-myc and max proteins were obtained from Dr. H. Kondo.

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