Minichromosome Maintenance Protein MCM7 Is a Direct Target of the MYCN Transcription Factor in Neuroblastoma

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ABSTRACT

The MYCN oncogene is amplified in ~25% of neuroblastoma tumors and is the most significant negative prognostic factor. The direct transcriptional targets of MYCN in MYCN-amplified tumors have not been defined. Microarray analysis of RNA from neuroblastoma primary cell cultures revealed 10-fold higher MCM7 expression in MYCN-amplified versus nonamplified tumors. MCM7 is an essential component of DNA replication licensing factor, a hexameric protein complex that regulates DNA synthesis during the cell cycle, preventing rereplication and ensuring maintenance of DNA euploidy. Additional experiments demonstrated markedly increased expression of MCM7 RNA and protein in MYCN-amplified neuroblastoma tumors and cell lines. Induction of MYCN in conditional cell lines results in increased expression of endogenous MCM7 mRNA and a 3-fold increase in protein levels. In addition, luciferase activity from MCM7 promoter/luciferase gene reporter constructs was significantly increased under MYCN-induced conditions. Specific electrophoretic mobility shifts of MCM7 promoter sequences are detected in extracts of MYCN-amplified cells. These findings demonstrate that in neuroblastoma, the MYCN oncogene directly activates genes required for DNA replication, and this may contribute to neoplastic transformation of these MYCN-amplified tumors.

INTRODUCTION

The MCM molecules are a highly conserved group of DNA-binding proteins with a vital function of “licensing” DNA synthesis during the transition from G1 to S phase of the cell cycle. Originally characterized in yeast, this group of proteins is required for activation of autonomous replicating sequences and progression through the cell division cycle. In eukaryotes, MCM2 to MCM7 are sequentially assembled into a heteromeric hexamer, the replication licensing factor, that binds to DNA replication origins after the origin recognition complex has assembled at the end of G1 (2). (For detailed reviews of MCM structure and function see Refs. 3–5.) MCM-mediated regulation of DNA synthesis ensures that DNA replicates only once during each cell cycle and is essential for maintaining euploidy. Immunohistochemical studies in a variety of tissues demonstrate the increased expression of MCM2, 5, and 7 in solid tumors and preneoplastic proliferative states (6–8). Their vital role in the maintenance of chromosomal integrity in normal cells makes the MCM molecules potential targets of the transforming effects of cellular oncogenes.

The promoter regions of MCM5, 6, and 7 each contain numerous E2F transactivation sites, suggesting that the E2F transcription factor may be primarily responsible for the coordinated increase in MCM mRNA noted at the G1-S boundary (9, 10). However, in addition to the E2F sites, the MCM7 promoter has an E-box binding site for the MYC oncoproteins (10). On the basis of initial observations demonstrating differential expression of MCM7 between MYCN-amplified and nonamplified neuroblastoma tumor cell cultures (see below), we pursued the hypothesis that MCM7 may be a direct target of MYCN.

MYCN, a close homologue of MYCC, functions as a transactivator by binding to a core E-box promoter element CAC/TGTG after forming a heterodimer with MAX. Although MYCC, MYCN, and MYCL can all bind to the canonical core element, promoter-flanking sequences can markedly affect binding affinities of the different MYC isoforms to individual target genes (11). In addition, other regulatory molecules that influence both the activity of MYC and the interaction of MYC with MAX contribute to the tissue specificity of MYCN and MYCC oncogenic transactivation (12, 13).

The MYCN proto-oncogene is amplified in ~25% of neuroblastomas. It is also frequently amplified in retinoblastomas, astrocytomas, gliomas, and small cell lung cancers. Amplification is the most reliable negative prognostic factor in neuroblastoma (14). Three-year survival for patients with metastatic neuroblastoma decreases from ~40% to <10% with MYCN amplification (15). Despite intensive efforts, MYCN transcriptional targets responsible for the particularly malignant phenotype of these tumors have remained elusive (16).

This report characterizes the direct interaction of MYCN with the MCM7 E-box, and consequent increased MCM7 mRNA and protein expression in MYCN-amplified cell lines and tumors relative to their nonamplified counterparts. We demonstrate consistent MYCN-dependent differential expression of MCM7 by direct immunohistochemistry in primary tumor samples and by conditional expression of MYCN in a neuroblastoma cell line. Transcriptional regulation of MCM7 by MYCN may have important implications for the pathogenesis, progression, and treatment of neuroblastoma and other MYCN amplified tumors.

MATERIALS AND METHODS

Tissues and Cell Lines. The neuroblastoma primary tumor cultures (P4, P46, P67, and P102) were established as described previously (17) and cultured in MEM with 10% FCS and antibiotics. The MYCN-inducible neuroblastoma cell line TET-21 derived from the SH-EP line was a kind gift from Dr. Manfred Schwab. These cells were grown as described previously in 10% serum/RPMI 1640 with penicillin and streptomycin (18). Tetracycline was used at 1 μg/ml concentration. IMR-32 and SK-N-SH cell lines were obtained from the American Type Culture Collection and grown as above. The Texas Children’s Hospital Department of Pathology supplied neuroblastoma tumor tissue for immunohistochemistry according to Institutional Review Board-approved protocols. This tissue was drawn from archived specimens that remained after diagnostic studies and would have otherwise been discarded.

Microarray Probing. Total RNA was extracted from exponentially growing cultures using the RNeasy Mini kit (Qiagen) according to the instructions of the manufacturer. Poly(A)+ RNA was purified using the oligo(dT) mRNA Mini kit (Qiagen). Each oligo(dT)-selected mRNA (200 ng) was reverse transcribed with Moloney murine leukemia virus reverse transcriptase enzyme (Promega). Random nonomers with fluorescent tags (CY3 or CY5) were used to prime each reaction and were carried out with standard dideoxy nucleotides. After transcription, the CY3 or CY5 samples were combined with prepared controls, purified over a
TE-30 spin column (Clontech), and concentrated. UniGem V microarrays (Incyte Genomics) were hybridized with two different fluorescent-labeled probes (CY3 and CY5). The probes were simultaneously applied to the microarray for competitive hybridization. After scanning and image analysis, a ratio of the two fluorescent intensities provided a quantitative measurement of relative gene expression levels. Only genes that showed significant expression in at least one channel were considered for calculation of differential expression. No differential expression was calculated for any element of which the signal did not measure ≥2.5 times the background signal in at least one channel. For our analyses, we used a background subtraction level of 200 relative fluorescent units. Identity of spotted cDNA was verified by PCR. A failure in PCR testing resulted in exclusion of the spot measurement. A total of 6800 gene products (cDNAs) passed these quality control criteria. Of these, 370 showed ≥3.5-fold differential expression for one of the tumor cell cultures.

cDNA Northern. cDNA Northern were used in this study to maximize detection sensitivity for MCM7 and other differentially expressed messages. This method has been demonstrated to accurately reflect expression levels and to correlate well with standard Northern blots (19). Total RNA from the induced and noninduced TET-21 cell line was isolated with the Qiagen RNeasy kit and amplified fragments (MYCN and H9262) were hybridized with 1.4% agarose gels to prevent over-cycling per protocol. Next, 1.5 μg of MYCN-induced and noninduced cDNA was electrophoresed and electroblotted onto nylon membranes. Probes for MYCN and MCM7 were generated from PCR amplified fragments (MYCN: 5'-CCTGCGCCGCCGCTGC-3' and 5'-CTCGTGGACTGAGCCA-3'; MCM7: 5'-AGCAGAACAATACGCTAGC-3' and reverse 5'-CCCTCTTGTCTCCTAGAAGAG-3') and either random hexamer labeled with [α-32P]dCTP or labeled with alkaline phosphatase (Amer sham Alkphos Direct). Probes were hybridized at 42°C in UltraHyb (Ambion) hybridization buffer or alkaline phosphatase hybridization buffer (Alkphos Direct kit, Amersham) overnight and washed. Probe signals were detected using a Molecular Dynamics PhosphorImager. Expression levels were normalized against the signal from β-actin.

Electrophoretic Mobility Shift Assays. Nuclear extracts were prepared as follows from logarithmically growing cell cultures. Cell pellets were solubilized in lysis buffer without NaCl [20 mM HEPES (pH 7.4), 3 mM MgCl2, 0.2 mM EDTA, 10% glycerol, and protease inhibitors (EDTA free complete; Roche)] on ice then centrifuged 15 min at 4°C. The resulting pellet was washed twice in lysis buffer and resuspended in lysis buffer plus 400 μM NaCl. Protein content was measured by the Bradford method (Bio-Rad) and salt concentration adjusted to 150 mM for binding assays. Oligonucleotide EMSA probes were generated by annealing commercially prepared sense and complement sequences followed by end-labeling with [γ-32P]ATP and T4 polynucleotide kinase. Segments (34-bp) of the MCM7 promoter containing the wild-type and mutated E-box sequences with the following sequences were used (sense strand with E-box region underlined, mutant bases italicized): wild-type = 5’-CGG AGT GGC GGC GGG GGT CAC CTG GGG GGC GAC GTT T-3’, mutant 1 = 5’-CGG AGT GGC GGC GGG GGT CAT ATG GGG GGC GAC GTT T-3’, mutant 2 = 5’-CGG AGT GGC GGC GGG GGT CAT ATG GGG GGC GAC GTT T-3’. Probe (1 μl; 105 cpm) was mixed with 5 μl of binding buffer (50 μg/ml random hexamer p(dN)6, 2.5 mg/ml BSA, 4% Ficoll 400, and 10% glycerol) and incubated with 10 μl of lysate (7 mg) after dilution to 150 mM NaCl and addition of 2 μg poly(dI-dC), for 30 min at 4°C. Competitors and antibodies were preincubated with the nucleic acid before adding poly(dI-dC) for 30 min before the addition of probe. Reactions were loaded onto 6% native acrylamide gels and electrophoresed on prerun gels for 1 h at 250 V at 4°C. Antibodies (sources) were as follows: polyclonal anti-MYC (C-19; Santa Cruz Biotechnology Research), monoclonal anti-MYC (AB-1; Oncogene Research), anti-MAX (C-124; Santa Cruz Biotechnology Research), and anti-IgG FC control (005098; Jackson Immunoresearch).

Luciferase Reporter Assays. The luciferase reporter constructs used in this report were a gracious gift from Dr. Toluji Kiyono. The full 0.5 Kb MCM7 promoter contains several E2F binding sites and one E-box. In luciferase reporter assays, a promoter fragment (AB) containing the E-box and the 3' E2F transactivation site accounted for more than half of the activity of the full-length promoter (10). This construct and a control plasmid containing mutated E-box and E2F sites (84M) were transfected into TET-21 cells grown in the presence of tetracycline (MYCN noninduced) using DOTAP (gift from Dr. Nancy Templeton). Endotoxin-free plasmid preps of each construct were prepared and mixed with DOTAP to produce lipid/DNA complexes. For each experiment, the wells of a six-well plate with 70% confluent TET-21 cells were transfected with DOTAP/plasmid complexes prepared as described (20). To ensure equivalent transfection conditions between MYCN-induced and noninduced cells, sufficient DOTAP/plasmid complexes were prepared to transfect the entire plate (≤10 μg DNA/well) and were added to each well under identical conditions and incubated with serum-free medium for 3 h. Standard medium containing tetracycline and serum was then added, and the cells were grown for 36 h. Medium was exchanged, and tetracycline-free medium was added to half the wells to induce MYCN expression. Cells were lysed in reporter lysis buffer and luciferase activity measured after 10 h of induction per protocol (Promega luciferase assay kit). In pilot experiments cotransfection with a β-galactoside plasmid was used to confirm that transfection efficiency was uniform between wells and that reporter gene activity was proportional to total protein in the cell lysates (β-galactoside activity did not change on removal of tetracycline; data not shown). Subsequently, luciferase activity was normalized to total protein in each well (luciferase activity/μg protein).

Immunohistochemistry. MCM7 immunohistochemistry was performed on formalin-fixed paraffin tumor sections with a mouse monoclonal anti-MCM7 antibody at 1:200 dilution (Santa Cruz Biotechnology). Prepared sections were washed in Tris-EDTA, and endogenous peroxidase was blocked by standard protocols. Biotinylated primary antibody was applied for 25 min at room temperature. Sections were incubated with horseradish peroxidase/streptavidin (BioGenex) for 10 min at room temperature per standard methods. Counterstaining was done with hematoxylin and samples mounted with aqueous mounting medium (Dako Faramount). Negative (withholding primary antibody) and positive controls (antibody specific for neuron-specific enolase) were performed with each tissue specimen. No significant staining occurred without primary antibody. All of the cells with neuronal/neuroblast-like morphology stained positive with the neuron-specific enolase antibody.

RESULTS

MCM7 Expression Correlates with MYCN Amplification in Neuroblastoma Tumors. We performed a cDNA microarray-based analysis comparing mRNA expression between tumor explants from two INSS stage 4 neuroblastoma patients, P4 and P67. In the MYCN-amplified (P4) cells, MCM7 message was increased 10.4-fold over the level in the nonamplified (P67) tumor. Table 1 lists some of the genes associated with MYCN overexpression.

Differential Expression Is the Ratio of the Greater to the Lesser Signal after Correction for Background. The observed relationship between MYCN and MCM7 expression was additionally investigated by immunohistochemistry on tissue sections from 15 MYCN-amplified and 14 nonamplified INSS stage III and stage IV neuroblastoma tumors (as well as 4 mature ganglioneuromas, which were entirely negative for MCM7; not shown; Fig. 1; Table 2). An anti-MCM7 monoclonal antibody stained the majority of tumor cells positive with a well-defined nuclear staining pattern in the MYCN-amplified samples. By contrast, only scattered foci of positive staining were demonstrated in the MYCN nonamplified samples, although the overall proliferative fractions of the tumor samples were very similar (see Fig. 1 legend). These foci may represent cells with increased MYCN expression, although this study does not address this hypothesis. No MYCN-amplified tumors had <+++ staining, whereas no nonamplified tumor had >+++ staining. H&E staining and neuron-specific enolase staining demonstrated no gross histological differences between the amplified and nonamplified specimens tested (data not shown).

MCM7 Is Rapidly Up-Regulated by Inducible MYCN Expression. To eliminate a coincidental correlation between MYCN and MCM7 expression, this phenomenon was additionally analyzed using a MYCN nonamplified neuroblastoma cell line, SH-EP, stably transfected with the MYCN gene under the control of the rTet-inducible expression system (MYCN expression is induced on removal of tet-
Table 1  Comparison of mRNA hybridization signal intensity for selected genes between MYCN-amplified (P4) and non-amplified (P67) neuroblastoma tumor cell cultures

<table>
<thead>
<tr>
<th>Gene name</th>
<th>P4 signal</th>
<th>P67 signal</th>
<th>Differential expression</th>
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<tr>
<td>MYCN</td>
<td>7514</td>
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<td>Laminin</td>
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<td>Interferon-inducible protein 9–27 (Incyte PD:2902903)</td>
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<td>Integrin beta 1 (Incyte PD:417451)</td>
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<td>Cell division cycle 2, G1 to S and G2 to M (Incyte PD:1523795)</td>
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<td>Transforming growth factor beta (Incyte PD:2375329)</td>
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<td>CDC21 homolog, MCM4 (Incyte PD:103669)</td>
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Table 2  Summary of MCM7 immunohistochemistry

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<th>Tumor MCM7 INSS stage</th>
<th>Tumor MCM7 INSS stage</th>
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<td>S1</td>
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Fig. 1. MCM7 immunohistochemistry in neuroblastoma tumors. A and C, two representative MYCN-amplified tumors. B and D, two representative MYCN nonamplified tumors. Overall, tumor histology and architecture were similar as indicated by H&E and neuron specific enolase staining (data not shown). Proliferative fractions (percentage S + G2-M phase) of these four tumors, A-D, were all markedly elevated (24.0, 26.0, 31.7, and 14.1, respectively), and staining for the proliferation marker, Ki-67 antigen, was equivalent among all samples. This staining pattern was consistently found for all 29 tumor specimens analyzed.
used to study the effect of MYCN on MCM7 promoter activity. Fig. 3 presents the data from five independent experiments demonstrating significant increases in promoter activity on MYCN induction (removal of tetracycline) with a mean increase of 64% (P < 0.01). A control construct with a mutated E-box and proximal E2F sites (A8M) consistently gave <5% of the activity seen from the native E-box containing vector and showed no significant difference on MYCN induction. This increase in MCM7 promoter activity in these transient transfections is less than that demonstrated for the chromosomal promoter in tumors (10-fold). Previous studies have noted similar results when comparing episomal transcription from transiently transfected reporter constructs to expression of integrated chromosomal genes (21–23). These transient transfection assays additionally support the direct interaction of MYCN with the MCM7 promoter.

**MYCN Protein Binds Specifically to the MCM7 Promoter.** The human MCM7 promoter consists of a DNA sequence 5' to the coding region that contains several transcription factor-binding sites including three E2F sites, three GC boxes, and an E-box (10). EMSAs were performed to determine whether MYCN is capable of binding to the E-box within the MCM7 promoter sequence. Nuclear lysates from several MYCN-amplified and nonamplified neuroblastoma cell lines and tumor cell primary cultures were used in EMSA experiments with a 34-bp end-labeled probe containing the E-box site and flanking regions from the MCM7 promoter (see “Materials and Methods”). Fig. 4A demonstrates a specific complex of protein bound to oligonucleotide probe retarded relative to free probe on a native acrylamide gel. Nuclear lysates from MYCN-amplified cell lines and tumors (IMR-32 and P46) consistently showed much higher intensity bands than their nonamplified counterparts (SK-N-SH shown for example; Fig. 4B) likely reflecting increased MYCN/MAX heterodimer content of MYCN-amplified tumor lines.

Competition experiments using cold probes with wild-type and mutated E-box sequences were performed to test the sequence specificity of the EMSA. Fig. 4, A and C show pronounced inhibition of complex formation with wild-type sequence as the cold competitor. This inhibition is abrogated when bases within the E-box motif of the competitor oligonucleotide are altered (CACGTG to CATATC or CATATG). Furthermore, anti-MAX and anti-MYCN antibodies specifically interfere with protein/DNA complex formation as shown in Fig. 4D. This effect is seen whether the antibodies are added before or after probe and lysate are combined. There was no such effect with the irrelevant polyclonal anti-immunoglobulin G FC antibody. No super-shift of the protein/DNA complex could be detected with the antibodies used suggesting that the antibodies interfere with formation of the MYCN/MAX/promoter complex and/or do not bind to the intact protein/DNA adduct.

Taken together these results indicate that in neuroblastoma tumor samples, primary tumor cell cultures, and cell lines, high MCM7 expression correlates with overexpression of MYCN. EMSA studies showing specific interaction of MYCN with the MCM7 promoter E-box element, combined with the kinetics of MCM7 gene activation closely after that of MYCN, implicate MCM7 as a definite transactivation target for the MYCN transcription factor. Thus, direct binding of MYCN to the transactivation site in the MCM7 promoter is partially responsible for the increased expression of MCM7 observed in MYCN-amplified tumor
cells. These findings have important implications for the pathogenesis and biology of neuroblastoma as discussed below.

DISCUSSION

Neuroblastoma is clinically and biologically heterogeneous. MYCN-amplified tumors are particularly aggressive and respond poorly to therapy. Whereas no mechanism linking gene amplification to this distinct clinical phenotype has been established, the bulk of evidence, both with MYCN as well as MYCC, supports a model in which increased expression of the oncogene, either by amplification or deregulated transcription, directly increases tumor cell growth rate and metastatic potential (16). A direct correlation exists between MYCN expression levels and growth rate, and motility and cell cycle alterations in cell culture models (18, 24, 25).

In vitro studies have also demonstrated that MYCN affects adhesion, invasiveness, response to growth factors, and autocrine factor production (26, 27). Additional evidence of the primary transforming ability of MYCN comes from the demonstration that tissue specific overexpression of MYCN leads to a neuroblastoma-like malignancy in transgenic mice (28). Although MYCN has been well characterized as a transcriptional regulator with clear transforming ability, the transcriptional target(s) that are responsible for this activity are not understood (16, 28). Recently, Boon et al. (29) reported significantly enhanced expression of ribosomal proteins, as well as proteins involved in ribosome biosynthesis and protein synthesis in MYCN-amplified neuroblastoma tumors and a MYCN-conditioned cell line. The full characterization of the downstream genes activated (or repressed) by MYCN amplification should help elucidate the mechanism underlying the clinical phenotype of MYC-overexpressing tumors in general and may suggest novel therapeutic approaches.

We have demonstrated that the binding of the MYCN/MAX heterodimer to its promoter E-box activates MCM7. This result is consistent with the increased expression of MCM7 protein in a MYCN-inducible neuroblastoma cell line, as well as in MYCN-amplified tumor tissue samples and tumor cell primary cultures. Whereas previous reports have demonstrated an association between cell proliferation and expression of MCM molecules (6, 7), several observations suggest that the MYCN-dependent up-regulation of MCM7 in neuroblastoma tumor cells is at
least partially independent of other MYCN-induced proliferative changes. First, MCM7 is rapidly up-regulated on induction of MYCN expression in the TET-21 cell line, with a time course more consistent with direct transcriptional changes, rather than as a secondary effect of increased proliferation. Second, the proliferative index and G1-S fraction of cells derived from the MYCN-amplified and nonamplified tumor specimens illustrated in Fig. 1 are very similar (see legend, Fig. 1), implying that this broad range of MCM7 expression is largely independent of proliferation rate. Third, a previous study by Otbtani et al. (9) demonstrates that serum stimulation or forced exogenous E2F expression activates the coordinate expression of the MCM proteins at the G1-S boundary in rat embryonic fibroblasts. Human MCM5, 6 and 7, as well as mouse Mcm3, have functional E2F binding sites in their promoters suggesting that this transcription factor is partly responsible for the coordinate MCM protein response to progression through the cell cycle (9, 10). Thus MCM7 binding to the unique E-box of MCM7 likely increases its expression independent of the other MCM molecules.

The concept that MCM7 may play an important regulatory role in tumor cell biology arises from several recent findings of novel MCM7/protein interactions. Yeast two-hybrid experiments have demonstrated MCM7 binding to RB1-related protein p107, resulting in down-regulation of DNA replication when MCM7 is limiting (30) to the HPV E6 oncoprotein (31) and to FLH-2 (a heart-specific LIM-zinc finger protein also expressed in lung cancer and chronic myelogenous leukemia cell lines; Ref. 32). MCM7 interacts with MAT-I, a subunit of the cyclin-dependent kinase activating kinase that promotes the stability and activation of the CDK7-cyclin H complex (33). It is also a component of a DNA helicase/ATPase (Refs. 34, 35; composed of MCM4, 6, and 7) and associates with the RNA polymerase II holoenzyme complex (36). The biochemical and functional properties of MCM7 suggest a regulatory role for this protein in cell division cycle and RNA synthesis. As such, its transcriptional activation by the MYCN oncogene is remarkable. In concert with other MYCN-mediated effects, transcriptional activation of MCM7 could be a significant component of MYCN-induced tumorigenesis.

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