THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 7, Issue of February 13, pp. 5110–5117, 2004
Printed in U.S.A.

E2F and Sp1/Sp3 Synergize but Are Not Sufficient to Activate the *MYCN* Gene in Neuroblastomas*

Received for publication, May 7, 2003, and in revised form, October 24, 2003
Published, JBC Papers in Press, November 26, 2003, DOI 10.1074/jbc.M304758200

Christoph Kramps, Verena Strieder, Alexandra Sapetschnig, Guntram Suske, and Werner Lutz‡

From the Institute of Molecular Biology and Tumor Research, 35033 Marburg, Germany

Amplification of the *MYCN* gene, resulting in overexpression of *MYCN*, distinguishes a subset of neuroblastomas with poor prognosis. We recently identified *MYCN* as a target gene of the E2F transcription factors. Here we show that Sp1 and Sp3 cooperate with E2F-1 to activate the *MYCN* promoter. However, in a neuroblastoma cell line that does not express *MYCN*, overexpression of E2F-1 was not sufficient to activate the *MYCN* promoter even in the presence of trichostatin A and 5-aza-cytidine. This was because of a failure of E2F-1 to bind to the *MYCN* promoter in these cells, although access of E2F-1 to the inactive *MYCN* promoter was not blocked by a nucleosome. Differences in nucleosomal organization of the *MYCN* promoter in different cell lines did not correlate with gene activation *per se* but with the switch from basal to activated transcription. Binding of E2F and Sp1/Sp3 to the *MYCN* promoter *in vivo* correlated with acetylation of histones H3 and H4 and recruitment of RNA polymerase II and the protein acetyltransferase Tip60 but not with nucleosome remodeling. Our results define distinct chromatin states of the *MYCN* promoter, indicate that factors in addition to E2F and Sp1/Sp3 are required to activate *MYCN* in neuroblastomas, and provide evidence for a novel mechanism of controlling access of E2F to selected target genes.

The transcription factors encoded by the *MYC* genes control diverse tumorigenesis-relevant processes such as cell-cycle progression, growth factor dependence, and response to anti-mitogenic signals (for a review, see Refs. 1 and 2). Overexpression of one of the *MYC* genes as a result of chromosomal translocation, gene amplification, or loss of negative transcriptional control plays a prominent role in the etiology of many types of tumors (for a review, see Refs. 3 and 4). The *MYCN* gene is found amplified in several tumors of mostly neuroendocrine origin including about 25% of neuroblastomas, the most common solid tumor in childhood (for a review, see Refs. 5 and 6). Whereas many neuroblastomas regress spontaneously or can be cured with minimal therapy, *MYCN*-amplified tumors have a poor prognosis. The treatment of these patients has not improved significantly in the course of the last two decades. Recently, the comparison of the gene expression profiles of *MYCN*-expressing *versus* non-expressing neuroblastoma cells as well as *MYCN*-amplified *versus* non-amplified primary tumors have begun to address the functional consequences of the massive overexpression of *MYCN* resulting from gene amplification (7, 8).

* This work was supported by the Deutsche Forschungsgemeinschaft. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 49-6421-2865390; Fax: 49-6421-2865196; E-mail: lutz@imt.uni-marburg.de.

Several mouse models of Myc-induced tumorigenesis suggest that Myc is not only required for the initiation but also for the maintenance of the tumorigenic state supporting the value of the *MYC* genes as targets of tumor therapy (9–11). Roughly half of the drugs that are in clinical use currently act as inhibitors of enzymes. In transcriptional regulation, enzymes are involved mainly as components of signal transduction cascades that relay information to the promoter and as transcriptional co-regulators that modulate local chromatin structure either by covalent modification of histones or by an ATPase-dependent remodeling of nucleosomes (for a review, see Ref. 12). The efficacy of histone deacetylase inhibitors against leukemia has proven the principle of treating cancer by the selective pharmacological modulation of the transcription machinery (13). Application of this concept to the *MYCN* gene requires a detailed knowledge of the molecular basis of the transcriptional activation of *MYCN* in neuroblastomas.

Toward this goal, we have recently identified the activating members of the E2F family of transcription factors (E2F-1, E2F-2, and E2F-3) as regulators of *MYCN* expression in neuroblastomas (14). E2F proteins are important regulators of cell-cycle progression, and their activity is negatively controlled by the p16/Rb pathway, which is inactivated in the majority of human cancers (for a review, see Refs. 15 and 16). Although genetic defects of the p16/Rb pathway are rare in neuroblastomas, there is some evidence for a loss of normal control of E2F activity in neuroblastomas by epigenetic means (17).

In addition to E2F binding sites, the *MYCN* promoter contains several putative binding sites for Sp1 and related zinc finger transcription factors (18). One of these, a non-consensus binding site, the CT-box, was previously implicated in *MYCN* regulation based upon *in vivo* footprinting data (19). Sp1 and the closely related protein Sp3 are the major GC-box binding activities in nuclear extracts from most mammalian cells (for a review, see Refs. 20 and 21). Sp1 can affect promoter activity in a number of different ways. Sp1 can protect CpG islands from methylation (22), can mediate the constitutive association of the general transcription apparatus with the core promoter to allow rapid induction in response to signals (23), and is able to recruit various activating and repressing co-regulators to a target promoter including the protein acetyltransferases p300/CBP (24), the CRSP complex (25), the SNF/SWI complex (26), and histone deacetylases (27, 28).

Here we show that Sp1/Sp3 and E2F cooperate to activate the *MYCN* promoter in neuroblastoma cells. However, E2F proteins and Sp1/Sp3 are not sufficient to activate the *MYCN* gene. We provide evidence that an additional step is required early in the activation process to allow binding of E2F proteins and Sp1/Sp3.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfections, and Reporter Assays—The human neuroblastoma cell lines SH-EP, IMR-32, LA-N-5, and Kelly were a gift of Manfred Schwab (Deutsches Krebsforschungszentrum Heidelberg). SY5Y cells were obtained from American Type Culture Collection. Neuroblastoma cell lines were cultured as described (19). *Drosophila Schneider* cells were cultured and transfected as described (29). 200 nM 4-OHT,[1] 330 nM TSA, and 500 nM 5-aC were added to the cell culture medium.

For the generation of stable cell clones, SH-EP cells were transfected with pCMVE2F1-ER (kindly provided by Kristian Helin) in Dulbecco's modified Eagle's medium using *N*,*N*-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid-buffered saline-calcium phosphate precipitation and selected with 200 μg/ml of G418. Individual clones were checked for the expression of the fusion protein by Western-blotting with an E2F-1 specific antibody (sc-193, Santa Cruz Biotechnology). SDS-gel electrophoresis, transfer to polyvinylidene difluoride membranes, and detection were performed according to standard procedures. A Cdk2-specific antibody was used to control for equal loading. Most of the luciferase reporter constructs have been described (19). The mutation in CT-box 1 was introduced into the wild-type reporter construct using the QuikChange kit (Stratagene).

Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared following the procedure of Schreiber (30). electrophoretic mobility-shift assays were performed by pre-incubating 1–2 μg of nuclear extract with 1.5 μg of unspecific competitor poly(dI-dC) in a buffer containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9, 150 mM KCl, 1 mM dithiothreitol, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 8.5% glycerol for 10 min on ice. Subsequently, 0.1 ng of ^{32}P-labeled double-stranded oligonucleotide was added to a final volume of 20 μl, and samples were incubated for another 20 min on ice. For competition assays, unlabelled Sp1-specific distal GC-box (GC2) or unspecific HNF3 oligonucleotide was added in access. Sp1 and Sp3 antisera used for supershift assays were described previously (31). 1 μl of the appropriate antiserum was added to the binding reaction, and incubation was continued on ice for another 20 min. Samples were analyzed on 4% native polyacrylamide gels in 45 mM Tris, 45 mM boric acid, 1.6 mM EDTA. Gels were transferred to Whatman 3MM paper, dried under heat and vacuum, and exposed to x-ray films overnight. The sequences of the oligonucleotides used for band shifts are available upon request.

Retroviral Infection—Human SH-EP neuroblastoma cells were transfected with an expression plasmid encoding the ecotropic receptor, and G418-resistant cells were pooled for infection with ecotropic retroviruses containing pBABE-ER-E2F-3 (kindly provided by Kristian Helin). Recombinant retroviruses were generated using the Phoenix E cell line. Infected cells were selected with 2 μg/ml of puromycin and analyzed immediately without further passaging.

FACS Analysis—Cells were harvested, washed with PBS, fixed with 70% ethanol for 30 min, incubated with RNase A overnight, and stained with propidium iodide. Fluorescence of 50,000 events was measured on a FACSCalibur™ (BD Biosciences). All measurements were performed in triplicate.

Chromatin Immunoprecipitation Assay—The protocol for chromatin immunoprecipitation and the primers used have been described (14). PCR products were either visualized on an agarose gel or directly quantified on an ABI7000 using SYBR Green. In the latter case, the number of amplification cycles required to reach a threshold is referred to as the threshold cycle (Ct) value. The differences in Ct values (ΔCt) displayed in the bar charts (Figs. 2, 5) were obtained by running PCRs in duplicate (all results shown had an S.D. $\leq$ 0.6). The mean of the Ct value for each sample was then subtracted from the corresponding number obtained with the control antibody (α-Gadd-45). In cases where results from different chromatin preparations had to be compared with each other (clone 1A3 with and without 4-OHT; SH-EP *versus* SY5Y), Ct values were corrected for differences in the Ct values of the input samples. In addition, all samples were checked for unspecific precipitation of chromatin using control primers. The following antibodies were used: anti-Gadd-45 (sc-H165, Santa Cruz Biotechnology), anti-diacetylated histone H3 (06–599, Upstate Biotechnologies), anti-acetylated histone H4 (06–866, Upstate Biotechnologies), anti-Sp3 (sc-644, Santa Cruz Biotechnology), anti-RNA-polymerase II (8WG16, kindly provided by Dirk Eick), anti-Tip60 was a kind gift of Bruno Amati. The rabbit anti-Sp1 is a polyclonal antibody raised against the full-length protein (31), and the antibodies specific for E2F family members and pocket proteins have been

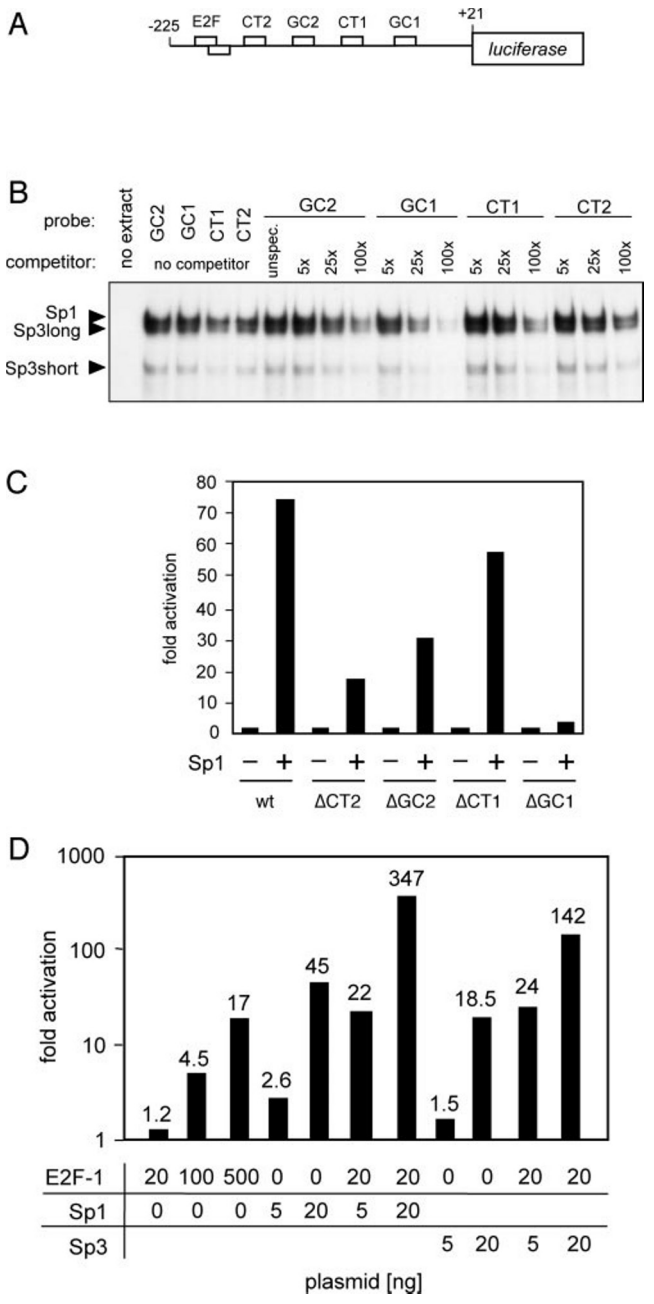

FIG. 1. **Sp1 and Sp3 activate the *MYCN* promoter and synergize with E2F-1.** *A*, schematic drawing of the *MYCN* reporter construct used. Luciferase expression is driven by 230 bp of the human *MYCN* promoter, including two overlapping E2F binding sites, four putative Sp1 binding sites (two GC-boxes and two CT-boxes), and the major transcription start sites. *B*, Sp1 and Sp3 bind to four sites in the *MYCN* proximal promoter *in vitro*. Electrophoretic mobility shift assays were performed with nuclear extract from the *MYCN*-amplified neuroblastoma cell line IMR-32 and radioactively labeled double-strand probes corresponding to the four putative Sp1/Sp3 binding sites present in the *MYCN* proximal promoter. The Sp1 binding site GC2 was used as an unlabeled competitor. The Sp1 and Sp3 proteins present in the complexes were identified by supershift assays (see Fig. 2*D*). *C*, all four binding sites contribute to the activation of the *MYCN* promoter by Sp1 and Sp3. Reporter constructs with clustered point mutations in each of the four Sp1/Sp3 binding sites were co-transfected along with Sp1 into *Schneider* cells. For each construct, luciferase activity in the absence of Sp1 was set to 1. *D*, Sp1/Sp3 and E2F-1 synergize to activate the *MYCN* promoter. *Drosophila Schneider* cells were transfected with 4 μg of the *MYCN* reporter construct and the indicated amounts of expression vectors.

[1] The abbreviations used are: 4-OHT, 4-hydroxy-tamoxifen; GC2, distal GC-box; Ct, threshold cycle; ChIP, chromatin immunoprecipitation; TSA, trichostatin A; 5-aC, 5-aza-cytidine.

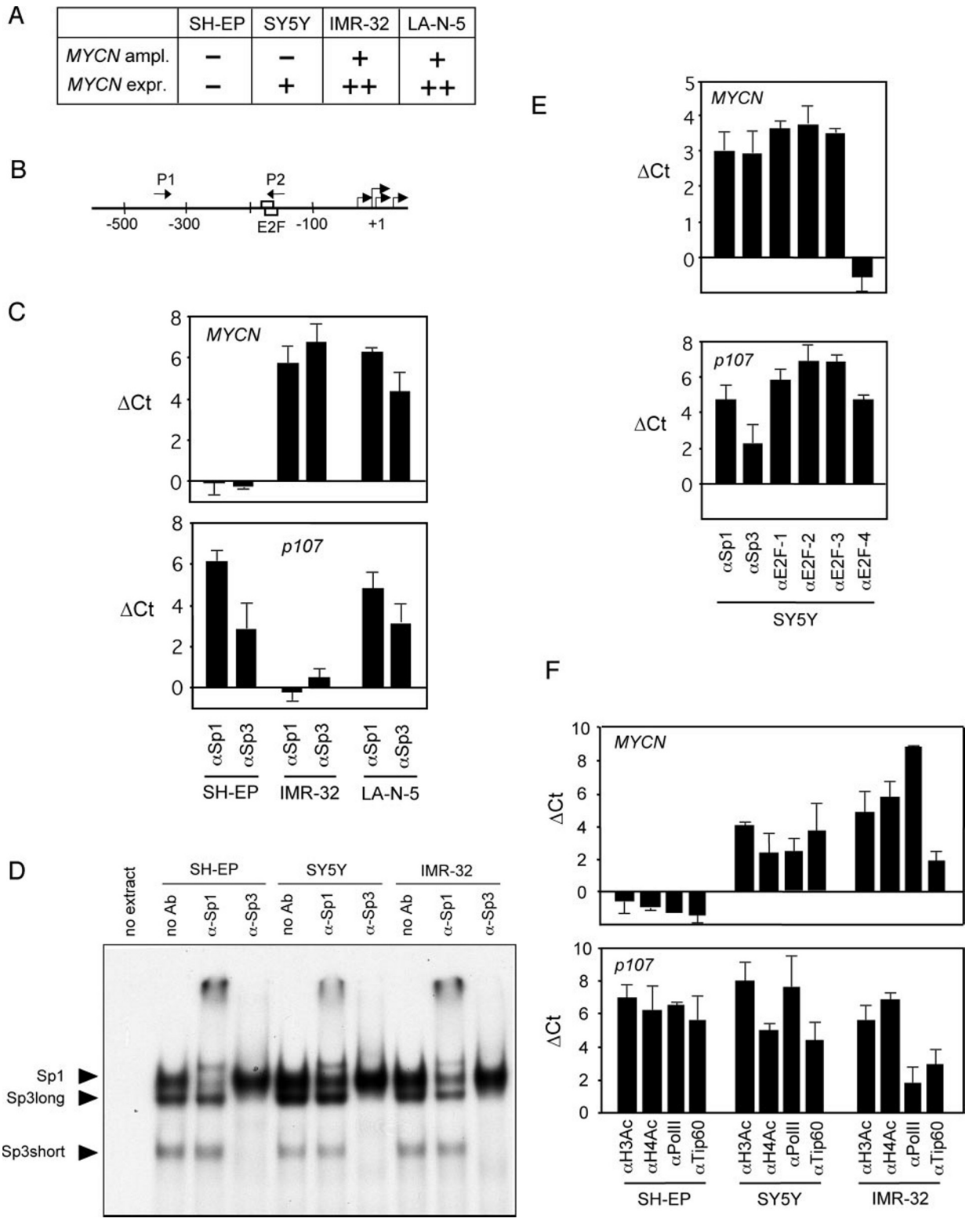

FIG. 2. **Sp1 and Sp3 bind the *MYCN* promoter *in vivo*.** *A*, status of the *MYCN* gene in the neuroblastoma cell lines used in this study. SH-EP is a neuroblastoma cell line lacking *MYCN* amplification and detectable *MYCN* expression. SY5Y is also single copy for *MYCN* but low levels of *MYCN* mRNA can be detected. IMR-32 and LA-N-5 are two neuroblastoma cell lines with *MYCN* amplification and strong *MYCN* expression. *B*, location of the primers used in the ChIP analyses of the *MYCN* promoter. The proximal promoter with the two overlapping E2F binding sites (*open boxes*) and the major transcription start sites (*arrows* around position +1) are shown. The four Sp1/Sp3 binding sites are located between the E2F binding sites and the transcription start sites (see Fig. 1*A*). The position of the primers used in the PCR with immunoprecipitated chromatin (designated P1 and P2) are indicated by *horizontal arrows*. *C*, Sp1 and Sp3 associate with the *MYCN* promoter specifically in neuroblastoma cells that express *MYCN*. Formaldehyde-crosslinked chromatin was precipitated with antibodies specific for Sp1 or Sp3 as indicated. PCRs were performed with primer pairs that amplify the promoter regions of *MYCN* and *p107* surrounding the E2F binding sites. Negative control reactions were performed without antibody and with an irrelevant control antibody (anti-Gadd45). The *bars* represent the difference between the mean Ct values of duplicate reactions obtained with the samples precipitated with the Sp1/Sp3 specific antibodies and the sample precipitated with the control antibody. *Bars*, S.D. for two independent experiments. *D*, Sp1 and Sp3 DNA-binding activity in nuclear extracts from neuroblastoma cell lines that differ in *MYCN* expression. Electrophoretic mobility shift assay was performed with the radioactively labeled GC2 probe and 2 μg of

used for chromatin immunoprecipitation experiments before (32). The IgG fraction was used for ChIP.

RT-PCR—Total cytoplasmic RNA was isolated with the RNeasy Kit (Qiagen) or TRIzol reagent, and 1 μg was reverse-transcribed using Superscript II and random primers. An aliquot of cDNA first strands corresponding to 50 ng of RNA was used for the PCR amplification. Prior pilot experiments established that the PCR conditions used are within the linear range of amplification. Primers amplifying part of the coding sequence of the small ribosomal subunit protein S14 were used to control for differences in RNA input. Primer sequences are available upon request.

Nucleosome Mapping—Nucleosomes were mapped using a published PCR-based procedure (33). Briefly, isolated nuclei were digested with 375 units of micrococcal nuclease (107921, Roche Applied Science) for 10 min at 37 °C. Mononucleosomal DNA was then gel-purified. 5 ng of mononucleosomal DNA and genomic DNA isolated from undigested nuclei in parallel were used as templates for PCR. Quantitative PCRs were performed on an ABI7000 using SYBR Green. The reactions were run in duplicate (for all data presented, the S.D. was $\leq$ 0.3 for Ct values). To exclude contamination of the mononucleosomal DNA with longer DNA fragments because of incomplete nuclease digestion, a PCR was performed with primers that amplify a 220-bp fragment from the *MYCN* promoter. DNA preparations were only used if the difference of Ct values between digested and undigested DNA was at least seven cycles. Primer sequences are available upon request.

RESULTS

Sp1 and Sp3 Activate the MYCN Promoter and Synergize with E2F-1—The proximal *MYCN* promoter contains two GC-boxes at positions −59/−64 and −126/−131 and two CT-boxes at positions −94/−99 and −155/−160. All four sites are putative binding sites for the transcription factors Sp1 and Sp3. To test whether Sp1 and Sp3 are able to activate the human *MYCN* promoter, expression vectors for Sp1, the long and short isoforms of Sp3, and E2F-1 were co-transfected with a luciferase reporter construct into *Drosophila Schneider* cells lacking endogenous Sp1 binding activity. The luciferase reporter gene is controlled by 230 bp of the human *MYCN* promoter including the E2F binding sites, the four putative Sp1/Sp3 binding sites, and multiple transcription start sites (Fig. 1*A*). Sp1 and the long isoform of Sp3 activated the *MYCN* promoter in a dose-dependent manner, whereas the short isoform of Sp3 that may function as a transcriptional repressor had no effect (data not shown). In electrophoretic mobility shift assays, Sp1 and Sp3 present in nuclear extracts of IMR-32 neuroblastoma cells that express *MYCN* bound to all four Sp1/Sp3 binding sites with similar affinity (Fig. 1*B*; the identity of the proteins producing the various shifts was confirmed with appropriate antibodies; see Fig. 2*B*). Consistent with this, clustered point mutations in any one binding site impaired promoter activation by co-transfected Sp1 (Fig. 1*C*). Mutation of the promoter proximal GC-box abolished activation of the promoter by Sp1 almost completely. Mutation of GC2 and the distal CT-box severely reduced promoter activity, whereas mutation of the proximal CT-box had only a mild but reproducible effect.

We previously showed that E2F-1 activates the *MYCN* promoter in neuroblastoma cells (14). Co-expression of either Sp1 or the long isoform of Sp3 with limiting amounts of E2F-1 resulted in synergistic activation of the *MYCN* promoter (Fig. 1*D*). Transfections with reporter constructs carrying mutations in individual binding sites showed that none of the Sp1/Sp3 binding sites on its own was essential for cooperation with E2F-1 (data not shown). In summary, Sp1 and Sp3 bind to four binding sites in the *MYCN* proximal promoter and synergize with E2F-1 to activate *MYCN*.

Sp1 and Sp3 Bind to the MYCN Promoter in Vivo—To test whether endogenous Sp1 and Sp3 bind to the *MYCN* promoter *in vivo*, chromatin immunoprecipitation (ChIP) assays were performed using antibodies against Sp1 and Sp3 and cross-linked chromatin from several human neuroblastoma cell lines differing in the status of the *MYCN* gene (Fig. 2*A*). IMR-32 and LA-N-5 show *MYCN* amplification and strong *MYCN* expression; SH-EP cells lack *MYCN* amplification, and *MYCN* expression is not detectable by RT-PCR. Precipitated chromatin was used as template for real-time PCR with primers that amplify a 200-bp fragment of the proximal *MYCN* promoter adjacent to the Sp1 binding sites (Fig. 2*B*). In IMR-32 and LA-N-5 cells expressing *MYCN*, both Sp1 and Sp3 were associated with the *MYCN* promoter. In contrast, in non-expressing SH-EP, neither Sp1 nor Sp3 were bound to the *MYCN* promoter (Fig. 2*C*). This was not because of inefficient cross-linking of Sp1/Sp3 to the chromatin in these cells, because interaction of Sp1 and Sp3 with the *p107* promoter, which contains a consensus Sp1 binding site, could readily be detected (Fig. 2*C*). To see whether differences in the amount of Sp1 and/or Sp3 in the nucleus are responsible for the absence of detectable binding to the *MYCN* promoter in SH-EP cells, electrophoretic mobility-shift assays were performed with nuclear extracts from the different neuroblastoma cell lines using GC2 as a probe. All extracts contained similar amounts of both Sp1 and Sp3 (Fig. 2*D*). Thus, the inactivity of the *MYCN* promoter in SH-EP cells does not result from a lack of Sp1 or Sp3 DNA-binding activity. We conclude that Sp1 and Sp3 bind to the *MYCN* promoter *in vivo* exclusively in neuroblastoma cells expressing *MYCN*.

We showed previously by ChIP analysis that E2F-1, E2F-2, and E2F-3 bind to the *MYCN* promoter in neuroblastoma cells with *MYCN* amplification *in vivo* (14). To analyze whether the regulatory interactions detected at the amplified gene also occur at a transcriptionally active single-copy *MYCN* gene, the ChIP experiment was performed with chromatin from SY5Y cells that lack *MYCN* amplification but express *MYCN* at a low level (34). Based on a quantitation of both *MYCN* mRNA levels and gene copy number using real-time PCR, we calculated that IMR-32 produces 3- to 4-fold more mRNA per gene copy than SY5Y (data not shown). As in *MYCN*-amplified cell lines, we detected binding of Sp1, Sp3, E2F-1, E2F-2, and E2F-3 to the *MYCN* promoter (Fig. 2*E*). E2F-4 did not interact with the *MYCN* promoter but was detected at the *p107* promoter. Thus, the same factors that regulate *MYCN* expression in cell lines with *MYCN* amplification also control expression of a single-copy *MYCN*.

Binding of E2F and Sp1/Sp3 Correlates with Histone Acetylation, Loading of RNA Polymerase II, and Recruitment of the Co-regulator Tip60—Transcription factors regulate gene expression in part by recruiting co-regulators, some of which change the acetylation status of nearby histones, to target promoters. E2F-1 has been shown to interact with the histone acetyltransferase p300/CBP and with TRRAP, a component of several histone acetyltransferase complexes that contain either Gcn5 or Tip60 as the enzymatically active subunit (35–37). However, recruitment of co-factors, histone acetylation, and loading of RNA polymerase can occur prior to the binding of a

nuclear extract from the neuroblastoma cells lines SH-EP, SY5Y, and IMR-32. Antibodies were added to the binding reactions as indicated to verify the presence of Sp1 and Sp3 in the shifted material. *E*, E2F proteins and Sp1/Sp3 bind to the promoter of a transcriptionally active single-copy *MYCN* gene *in vivo*. ChIP assays were performed with chromatin from SY5Y neuroblastoma cells that carry a single-copy *MYCN* gene and express low levels of *MYCN* mRNA with antibodies against Sp1, Sp3, E2F-1, E2F-2, E2F-3, and E2F-4. *F*, binding of E2F proteins and Sp1/Sp3 correlate with acetylation of histone H3 and histone H4 and recruitment of RNA polymerase II and the transcriptional co-regulator Tip60. ChIP assays were performed with antibodies recognizing acetylated histone H3 and histone H4, Tip60, and RNA polymerase II, and analyzed as described in the legend to Fig. 2*C*.

particular transcription factor (*e.g.* see Refs. 23 and 38). Therefore, we asked whether histone acetylation, loading of RNA polymerase, and recruitment of co-factors, exemplified by Tip60, occur at the *MYCN* promoter prior to the binding of E2F and Sp1/Sp3. ChIP assays showed that, in *MYCN*-expressing IMR-32 and SY5Y cells, histone H3 and histone H4 were acetylated, whereas in SH-EP cells lacking *MYCN* expression, they were not (Fig. 2*F*). Yet, acetylated histones could be detected at the *p107* promoter in all cell lines tested, including SH-EP. Likewise, RNA polymerase and Tip60 were detected at the *MYCN* promoter specifically in cells expressing *MYCN* but not in SH-EP cells (Fig. 2*F*). In contrast, the *p107* promoter that is bound by E2F in all cell lines analyzed was associated with Tip60 in all cell lines, including SH-EP, thus showing that cross-linking of chromatin had been as efficient in SH-EP cells as in the other cells lines. We conclude that binding of E2F and Sp1/Sp3 to the *MYCN* promoter correlates with local acetylation of histones and recruitment of RNA polymerase and the co-regulator Tip60.

E2F-1 Induces CCNE1 Expression and Apoptosis in Neuroblastoma Cells but Is Not Sufficient to Activate MYCN—Inhibition of E2F activity by overexpression of p16Ink4A reduces *MYCN* expression in neuroblastoma cells, suggesting that E2F is necessary for full activity of the *MYCN* promoter (14). Is E2F-1 in combination with endogenous Sp1/Sp3 also sufficient to activate the *MYCN* gene in a non-expressing cell line? To address this question, we overexpressed a 4-hydroxy-tamoxifen (4-OHT)-regulated E2F-1-ER fusion protein in SH-EP cells that do not express *MYCN* and in which endogenous E2F proteins cannot be detected by ChIP at the *MYCN* promoter *in vivo* (14). Treatment of a representative clone expressing high amounts of the fusion protein, SHEP-1A3, with 4-OHT for 12 h did not induce *MYCN* expression (Fig. 3*A*, compare *lanes 4* and 7). In contrast, expression of *CCNE1*, another E2F target gene encoding cyclin E, was induced after treatment with 4-OHT (Fig. 3*A*). In addition, the gene coding for inhibin βA (*INHBA*), a secreted glycoprotein involved in signaling through transforming growth factor-β superfamily members, which was previously shown to be repressed by E2F-1 in U2OS osteosarcoma cells (39), was down-regulated (Fig. 3*A*). Moreover, the cells underwent massive cell death after treatment with 4-OHT (Fig. 3*B*). After 24 h of 4-OHT treatment, almost 20% of the population displayed a sub-diploid DNA content representing cells that undergo apoptosis. At this time, 35% of the 4-OHT-treated cells were in S phase, compared with only 14% in ethanol-treated controls, suggesting that ectopic E2F-1 not only induced cell death but also stimulated entry into S phase (data not shown). After 36 h, roughly 35% of the cells were apoptotic, and after 48 h, virtually all cells had died. From these data, we conclude that the E2F-1-ER fusion protein is functional in SHEP-1A3 cells.

In transient assays in neuroblastoma cells, we had observed that treatment with trichostatin A (TSA), an inhibitor of histone deacetylases, increased expression of a *MYCN* promoter-driven reporter construct.[2] Therefore, we treated SHEP-1A3 cells simultaneously with 4-OHT and TSA, and also with 5-azacytidine (5-aC), an inhibitor of DNA methyltransferases. Simultaneous treatment of cells with both drugs can derepress a number of otherwise silent genes by preventing the establishment and maintenance of a repressive chromatin structure (*e.g.* see Refs. 40 and 41). We reasoned that by removing chromatin modifications associated with repressed genes, E2F-1 might become able to activate *MYCN*. However, a combination of 4-OHT, 5-aC, and TSA did not result in detectable activation of the endogenous *MYCN* in SH-EP (Fig. 3*A*, compare *lane 4* with *lane 12*). Furthermore, neither 5-aC nor TSA could prevent the repression of *INHBA* by E2F-1 (Fig. 3*B*). In a parallel experiment, we observed derepression of the *CDKN2A* gene by 5-aC in HeLa cells and stimulation of a reporter gene by TSA, demonstrating that the 5-aC and TSA used were active (data not shown).

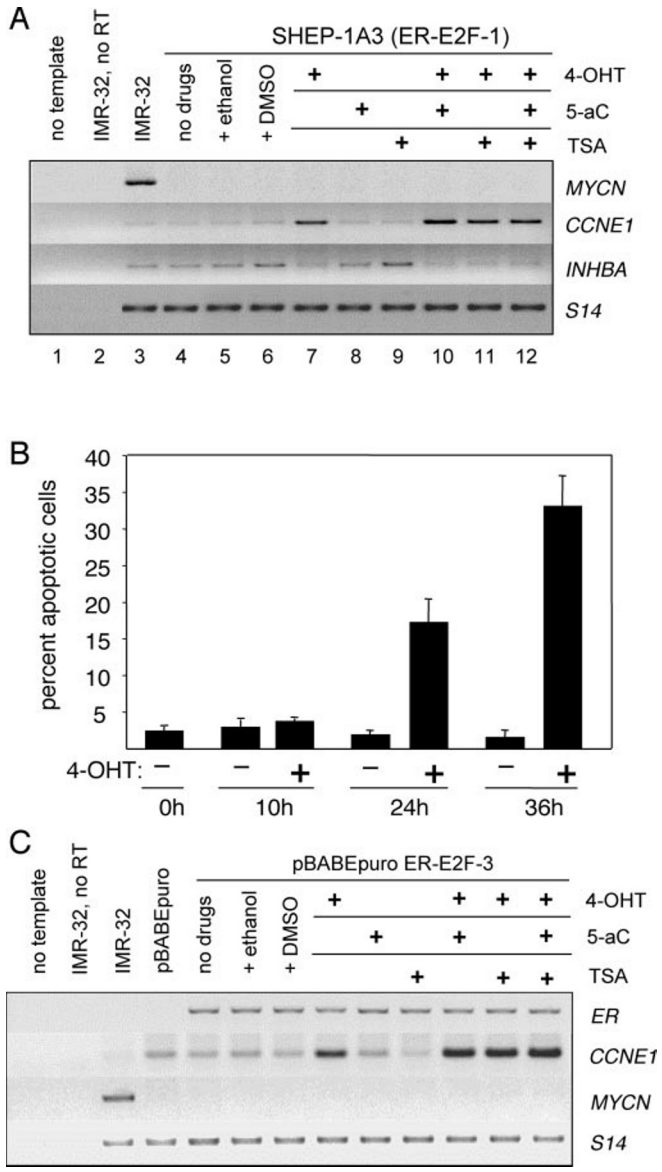

FIG. 3. **Overexpression of E2F-1 or E2F-3 is not sufficient to activate the *MYCN* gene.** *A*, an E2F-1-ER fusion protein regulates E2F target genes but not *MYCN*. SHEP-1A3, a clone of SH-EP that stably expresses a 4-OHT regulatable E2F-1-ER fusion protein, was treated with 4-OHT alone, a combination of 4-OHT with 5-aC, and/or TSA, or the solvent controls. 5-aC was added 36 h prior to harvest; 4-OHT and TSA were added 12 h prior to harvest. RNA was isolated, and RT-PCR was used to detect the mRNAs of *CCNE1*, *INHBA*, *MYCN*, and *S14* encoding the small ribosomal subunit S14. *B*, overexpression of E2F-1 triggers apoptosis in neuroblastoma cells. SHEP-1A3 cells were treated with 4-OHT or the solvent control ethanol for the indicated times. Cells were then fixed, stained with propidium iodide, and analyzed by FACS. The fraction of cells with a sub-diploid DNA content is shown as a percent of total cells analyzed. *Bars*, S.D. obtained with triplicate samples. *C*, E2F-3-ER induces *CCNE1* but not *MYCN*. SH-EP cells expressing the ecotropic receptor were infected with an ecotropic retrovirus carrying an E2F-3-ER fusion gene. The infected cells were selected for 2 days with puromycin and then treated with different combinations of 4-OHT, TSA, and 5-aC. Cells were harvested and RNA was isolated. Expression of the E2F-3-ER fusion gene, *CCNE1*, *MYCN*, and *S14* were analyzed by RT-PCR.

[2] C. Kramps and W. Lutz, unpublished observations.

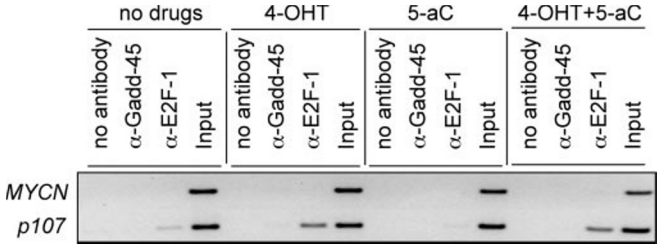

FIG. 4. **E2F-1 does not bind to the promoter of a silent *MYCN* gene.** SHEP-1A3 cells were grown in medium containing either solvent control, 4-OHT (for 15 h), 5-aC (for 36 h), or a combination of 4-OHT and 5-aC. Cross-linked chromatin was isolated from the cells and precipitated with an E2F-1-specific antibody or the Gadd-45 control antibody. Purified chromatin was amplified with promoter-specific primers for *MYCN* and *p107*.

In transient assays in SH-EP cells, E2F-3 is a stronger activator of the *MYCN* promoter than E2F-1 (14). Therefore, pools of SH-EP cells expressing the ecotropic receptor were infected with an ecotropic retrovirus encoding a 4-OHT-regulated E2F-3-ER fusion protein (42). Infected cells were selected with puromycin for 2 days and were then treated with combinations of 4-OHT, 5-aC, and TSA prior to isolation of RNA. E2F-3, like E2F-1, induced *CCNE1* and repressed *INHBA* but was unable to activate the *MYCN* gene alone or in combination with 5-aC and TSA (Fig. 3*C*). We conclude that overexpression of E2F-1 or E2F-3 in combination with endogenous Sp1/Sp3 is not sufficient to induce *MYCN* expression in neuroblastoma cells even when enzymatic activities with a repressive effect on transcription are inhibited.

E2F-1 Cannot Bind the Promoter of a Transcriptionally Inactive MYCN Gene—Why are E2F-1 and E2F-3 unable to activate a silent *MYCN* gene? The sequence of the proximal promoter of the *MYCN* gene, including all E2F and Sp binding sites, is identical in SH-EP and IMR-32 cells (data not shown). This leaves two explanations for the failure of overexpressed E2F-1 and E2F-3 to activate the *MYCN* gene. Either E2F proteins are unable to bind to their binding sites in the *MYCN* promoter in SH-EP cells, or they can bind but, on their own, are not capable of activating the gene. To distinguish between these alternative explanations, ChIP assays were performed with SHEP-1A3 cells expressing the 4-OHT-regulated E2F-1-ER fusion protein. As previously observed in the parent cell line SH-EP (14), an antibody specific for E2F-1 precipitated the E2F-regulated *p107* promoter but not the *MYCN* promoter (Fig. 4). Activation of the exogenous E2F-1-ER fusion protein by 4-OHT increased binding of E2F-1 to the *p107* promoter, demonstrating that the fusion protein is capable of binding to DNA in these cells. A 4-OHT-dependent increase in the binding of E2F-1 was also shown for other genes, including *CDC6* and *E2F1* (data not shown). However, no binding of E2F-1-ER to the *MYCN* promoter could be detected (Fig. 4). CpG methylation has been shown to prevent binding of E2F proteins to some E2F binding sites (43, 44). Therefore, we pre-treated 1A3 cells with the DNA methyltransferase inhibitor 5-aC to remove repressive promoter-methylation prior to the addition of 4-OHT. But even treatment of cells with 5-aC did not result in detectable binding of E2F-1-ER to the *MYCN* promoter. We conclude that E2F-1 cannot activate a silent *MYCN* gene in SH-EP cells because of its inability to bind to the *MYCN* promoter.

Nucleosomal Organization of the MYCN Promoter—What prevents binding of E2F proteins to the promoter of a transcriptionally silent *MYCN* gene? Previous *in vivo*-footprinting experiments did not provide evidence for the presence of repressive proteins at or near the E2F binding sites of the *MYCN* promoter in SH-EP cells (19). Alternatively, a nucleosome positioned over the E2F binding sites might prevent binding of

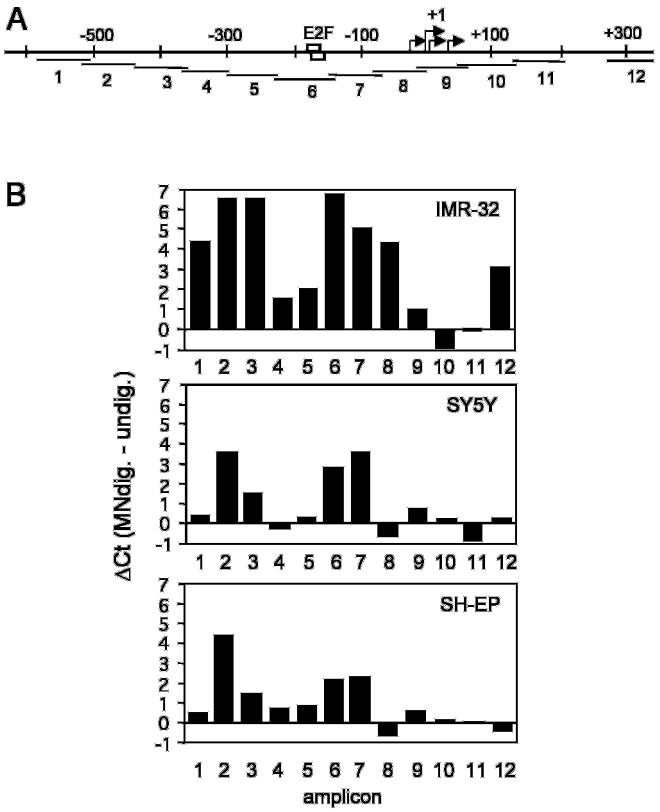

FIG. 5. **Positions of nucleosomes in the *MYCN* promoter.** *A*, location of the amplicons used for the mapping of nucleosomes at the human *MYCN* promoter (position −550 to +300). *Open boxes* indicate the two overlapping E2F binding sites; *arrows* mark the transcription start sites. *B*, access of micrococcal nuclease to the *MYCN* promoter. Nuclei were incubated with micrococcal nuclease to digest internucleosomal DNA. Real-time PCRs were performed with 5 ng each of undigested and mononucleosomal DNA using the primer pairs that amplify the fragments depicted in Fig. 5*A*. The reduction in signal intensity by nuclease treatment for each primer pair is represented as the difference in the Ct values between the reactions with nucleosomal and undigested DNA (mean Ct nucleosomal DNA − mean Ct undigested DNA).

E2F to the *MYCN* promoter. Therefore, accessibility of the E2F binding sites to micrococcal nuclease was analyzed in several neuroblastoma cell lines using a PCR-based method (33). Mononucleosomal DNA was generated by micrococcal nuclease digestion of nuclei and amplified by real-time PCR using a set of primer pairs that generate overlapping amplicons with an average size of 90 bp (Fig. 5*A*). PCR-amplification with a particular primer pair will be equally efficient with undigested and digested DNA if the region to be amplified was protected from nuclease digestion by being part of a nucleosome. In contrast, if the region to be amplified was accessible to micrococcal nuclease, then digested DNA will be amplified less efficiently than undigested DNA. Thus, the bigger the difference in Ct values between undigested and digested DNA, the more accessible the corresponding promoter region was to micrococcal nuclease. In IMR-32 cells, a region surrounding the multiple transcription start sites (primer pairs 9, 10, and 11) was protected from digestion (Fig. 5*B*). The protected region spans 230 bp, being considerably larger than the 146 bp wrapped around a nucleosome core, indicating either variation in the precise position of the nucleosome within the cell population or protection of this region by a multiprotein complex instead of a nucleosome. The region protected from nuclease digestion was extended upwards in both SH-EP and SY5Y, including the most proximal Sp1/Sp3 binding site (in addition to primer pairs 9–11, primer pair 8 amplifies a product equally efficient with nuclease-digested DNA and undigested DNA). The region amplified by

primer pair 12 was protected in SH-EP and SY5Y, but not in IMR-32, suggesting that the nucleosomal region extends further into exon 1 in SH-EP and SY5Y, but not in IMR-32. A region of 150 bps upstream of exon 1 (including the E2F binding sites and all but the most proximal of the Sp1/Sp3 binding sites) was sensitive to nuclease digestion in all three cell lines (primer pairs 6 and 7). The Ct difference between undigested and nuclease-digested DNA using primer pairs 6 and 7 was 2 to 3.5 in SY5Y and SH-EP, corresponding to a 4- to 8-fold difference (this is what has been obtained for other nucleosome-free loci with the same technique (45). In the case of IMR-32, the Ct difference between undigested and mononucleosomal DNA was larger for these same primer pairs perhaps because of the 100-fold amplification of the *MYCN* locus in these cells. The nucleosome-free region is flanked by a nucleosome around position −300 in all cell lines (primer pairs 4 and 5). In SH-EP and SY5Y, but not IMR-32, there is an additional protected region further upstream (primer pair 1). In summary, there are no differences in the nucleosomal organization of the *MYCN* promoter in SY5Y and SH-EP cells, although the *MYCN* gene is transcriptionally active only in SY5Y. In contrast, IMR-32 cells lack several of the protected regions observed in SY5Y and SH-EP (primer pairs 1, 8, and 12). However, the E2F binding sites are accessible to nuclease in both *MYCN*-expressing cells and non-expressing SH-EP, suggesting that the inability of E2F-1-ER to bind to the *MYCN* promoter in clone SHEP-1A3 is not due to masking of the E2F binding sites by a positioned nucleosome.

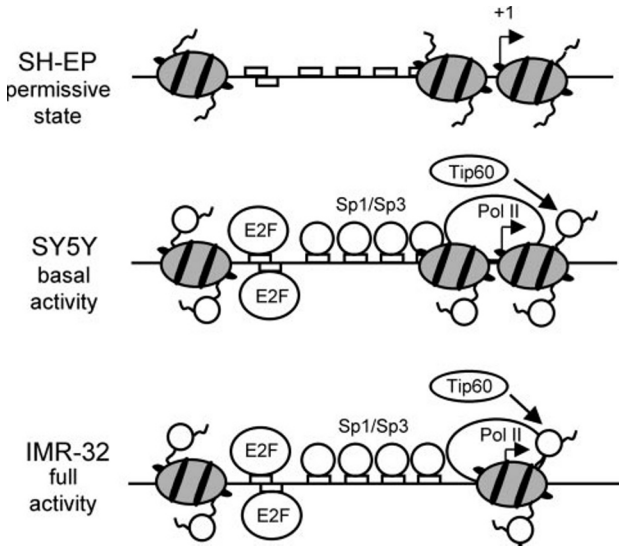

FIG. 6. **Model of the chromatin states of the *MYCN* promoter in different neuroblastoma cell lines.** The *MYCN* promoter is "frozen" in different chromatin states in different neuroblastoma cell lines reflecting the differential activity of the *MYCN* promoter in these cells (permissive, basal activity, full activity). The *arrow* indicates the major transcription start site; the *open boxes* represent binding sites for E2F and Sp1/Sp3; the *open circles* represent acetylation of the amino termini of histone H3 and histone H4; nucleosomes are indicated as *gray shapes* with protruding amino termini of histones.

DISCUSSION

The results presented here and in a previous report (14) begin to reveal the regulation of the *MYCN* promoter in neuroblastomas. The finding that Sp1 and Sp3 contribute to the activation of *MYCN* in neuroblastomas together with the recent identification of E2F-1, E2F-2, and E2F-3 as activators of *MYCN* puts *MYCN* into a group of genes that are regulated by the cooperation of Sp1/Sp3 and E2F. Included in this group are genes that share a role in the cell cycle, such as *cyclin D1* and *dhfr* (46, 47). Several lines of evidence suggest that cooperation between E2F and Sp1 is required to achieve cell cycle-dependent regulation of these target promoters, entailing both an activating mode during late G_1 and S phases and a histone deacetylase-dependent repressing mode during G_0 and early G_1 (27, 47, 48). Sp1 and E2F cooperate by means of direct physical interaction (49). The cooperation at the murine *TK* promoter is impaired when the distance between the Sp1 and E2F binding sites is increased, indicating that a precise spatial arrangement of factors is required for cooperation (50). In the *MYCN* promoter, the two overlapping E2F binding sites and the nearest Sp1 binding site, distal CT-box, show the same spacing of 9 bp that is critical for the cooperation at the murine *TK* promoter.

Based upon the lack of binding of both E2F-1 and Sp1/Sp3 to the inactive *MYCN* promoter in SH-EP cells, we conclude that neither of these transcription factors is involved in repressing *MYCN*. Instead, they seem to function exclusively in the activation of the *MYCN* gene. However, the presence of E2F and Sp1/Sp3 in cells is not sufficient to activate a transcriptionally silent *MYCN* gene. This is a surprising result given that the transactivation domain of E2F-1 can stimulate both transcriptional initiation and elongation (51) and can confer cell-cycle control when brought to a promoter by the Gal4 DNA-binding domain (48). In fact, based on this observation, it was proposed that cooperating transcription factors such as Sp1 are mainly required to achieve stable interaction of E2F with the target promoter (48). This raises the question of why overexpressed E2F-1 in combination with endogenous Sp1/Sp3 failed to bind to the inactive *MYCN* promoter, while at the same time it bound to other target genes.

One possibility is that CpG methylates the E2F binding sites, as this can affect DNA binding of E2F-1 and, at some promoters, also E2F-2 and E2F-3 (43, 44). However, methylation is unlikely to account for the failure of E2F-1 to bind to the *MYCN* promoter for several reasons. (*i*) CpG methylation leads to the recruitment of methyl binding proteins and the assembly of a repressive multiprotein complex. This complex triggers packaging of the chromatin, making the DNA inaccessible to transcription factors (for a review, see Ref. 52). Yet, the fact that micrococcal nuclease had access to the E2F binding sites argues against a condensed higher order structure of the *MYCN* promoter in SH-EP cells. Indeed, using chromatin immunoprecipitation, we did not detect methylation of histone H3 at Lys-9, a histone modification associated with heterochromatin, at the *MYCN* promoter in SH-EP cells (data not shown). Furthermore, by using *in vivo* footprinting, we previously did not detect proteins at the E2F binding sites in SH-EP cells (19). This observation also excludes competitive binding of a repressor protein in the vicinity of the E2F binding site as a reason for the inability of E2F to bind to the silent *MYCN* promoter. (*ii*) Even prolonged treatment of 1A3 cells with 5-aC before induction with 4-OHT did not result in binding of E2F-1-ER or activation of endogenous *MYCN*. (*iii*) In SH-EP cells, there was also no binding of Sp1 and Sp3 to the *MYCN* promoter, although binding of Sp1 is not affected by methylation of DNA (53). Because our data also exclude masking of the E2F binding sites by a positioned nucleosome, we propose that an unknown mechanism controls the binding of E2F and also Sp1/Sp3 to the *MYCN* promoter in neuroblastoma cells. The events that trigger binding of E2F to the *MYCN* promoter are an important issue, because they will not only provide an explanation for the maintenance of *MYCN* expression in neuroblastomas but will, in addition, reveal a mechanism for the tissue-specific modulation of the genetic program controlled by E2F.

MYCN is a neuroblastoma-specific target gene of both E2F and Sp1/Sp3. It is presently unclear if such tissue-specific

components of the genetic program controlled by E2F are common or if *MYCN* is an exception. *MYCN* expression is clearly not required for the induction of S phase or apoptosis by E2F-1, as both programs are triggered in SHEP-1A3 cells in the absence of *MYCN* activation. This points to novel cell-type-specific functions of E2F-1 and indicates that the consequences of E2F deregulation in neuroblastomas differ, at least in some aspects, from those observed in other tissues. The control of *MYCN* expression at the level of E2F binding is not observed at other E2F-regulated promoters in the same cells. Thus, in the future, it may be feasible to block activation of *MYCN* by E2F without affecting the expression of other E2F-regulated genes.

In the neuroblastoma cell lines analyzed, the *MYCN* gene is "frozen" in distinct chromatin states, representing different stages of promoter activation (Fig. 6). These distinct states have in common that a region containing multiple regulatory elements, including the E2F binding sites, is in an "open" configuration, as judged from nuclease accessibility. Thus, in SH-EP cells lacking *MYCN* expression, the *MYCN* gene presumably is in a "permissive" rather than a repressed state, which is consistent with the inability of TSA and 5-aC to derepress *MYCN* in these cells. The accessible region is flanked by nucleosomes, one of which overlaps with the transcription start site. This situation is reminiscent of the interferon-β promoter, where a similar nucleosome-free region binds multiple factors that assemble into a multiprotein complex, the enhanceosome (54). Because SY5Y cells (in contrast to SH-EP cells) show binding of E2F proteins to the *MYCN* promoter and *MYCN* expression, but the cell lines do not differ in nucleosomal organization of the *MYCN* promoter, repositioning of nucleosomes is not a prerequisite for binding of E2F and basal promoter activity. Despite differing in *MYCN* expression, SY5Y and SH-EP are two sub-clones of the same parental cell line SK-N-SH (55). SH-EP cells have an epithelial morphology, whereas SY5Y cells show signs of neuronal differentiation. SH-EP and SY5Y have been observed to transdifferentiate into each other at low frequency in culture (55). Their common ontogenetic history suggests that epigenetic events during early development do not determine transcriptional activity of the *MYCN* gene in neuroblastomas as has been proposed (56).

The binding of E2F proteins and Sp1/Sp3 to the *MYCN* promoter correlated with recruitment of Tip60 and histone acetylation. Because E2F-1 has been shown to interact with TRRAP, a subunit of several histone acetyltransferase complexes, E2F-1 may be responsible for the recruitment of Tip60 to the *MYCN* promoter (35, 36). In contrast, binding of E2F and Sp1/Sp3 did not correlate with the nucleosome remodeling that accompanies the switch from basal to full promoter activity. This suggests that full promoter activity requires a remodeling activity that is recruited to the *MYCN* promoter after binding of E2F and Sp1/Sp3.

Acknowledgments—We thank Kristian Helin for providing plasmids, Manfred Schwab for cell lines, Bruno Amati and Dirk Eick for antibodies, Iris Rohner for expert technical assistance, and Mary Lou Zouzarte for FACS analyses. W.L. is grateful to Martin Eilers for his support.

REFERENCES

1. Grandori, C., Cowley, S. M., James, L. P., and Eisenman, R. N. (2000) *Annu. Rev. Cell Dev. Biol.* **16,** 653–699
2. Lutz, W., Leon, J., and Eilers, M. (2002) *Biochim. Biophys. Acta* **1602,** 61–71
3. Eisenman, R. N. (2001) *Genes Dev.* **15,** 2023–2030
4. Henriksson, M., and Luscher, B. (1996) *Adv. Cancer Res.* **68,** 109–182
5. Brodeur, G. M. (2003) *Nat. Rev. Cancer* **3,** 203–216
6. Savelyeva, L., and Schwab, M. (2001) *Cancer Lett.* **167,** 115–123
7. Berwanger, B., Hartmann, O., Bergmann, E., Bernard, S., Nielsen, D., Krause, M., Kartal, A., Flynn, D., Wiedemeyer, R., Schwab, M., Schafer, H., Christiansen, H., and Eilers, M. (2002) *Cancer Cell* **2,** 377–386
8. Boon, K., Caron, H. N., van Asperen, R., Valentijn, L., Hermus, M. C., van Sluis, P., Roobeek, I., Weis, I., Voute, P. A., Schwab, M., and Versteeg, R. (2001) *EMBO J.* **20,** 1383–1393
9. Felsher, D. W., and Bishop, J. M. (1999) *Mol. Cell* **4,** 199–207
10. D'Cruz, C. M., Gunther, E. J., Boxer, R. B., Hartman, J. L., Sintasath, L., Moody, S. E., Cox, J. D., Ha, S. I., Belka, G. K., Golant, A., Cardiff, R. D., and Chodosh, L. A. (2001) *Nat. Med.* **7,** 235–239
11. Pelengaris, S., Littlewood, T., Khan, M., Elia, G., and Evan, G. (1999) *Mol. Cell* **3,** 565–577
12. Narlikar, G. J., Fan, H. Y., and Kingston, R. E. (2002) *Cell* **108,** 475–487
13. Redner, R. L., Wang, J., and Liu, J. M. (1999) *Blood* **94,** 417–428
14. Strieder, V., and Lutz, W. (2003) *J. Biol. Chem.* **278,** 2983–2989
15. Harbour, J. W., and Dean, D. C. (2000) *Nat. Cell. Bio.* **2,** E65–E67
16. Trimarchi, J. M., and Lees, J. A. (2002) *Nat. Rev. Mol. Cell Biol.* **3,** 11–20
17. Takita, J., Hayashi, Y., Nakajima, T., Adachi, J., Tanaka, T., Yamaguchi, N., Ogawa, Y., Hanada, R., Yamamoto, K., and Yokota, J. (1998) *Oncogene* **17,** 3137–3143
18. Strieder, V., and Lutz, W. (2002) *Cancer Lett.* **180,** 107–119
19. Lutz, W., and Schwab, M. (1997) *Oncogene* **15,** 303–315
20. Philipsen, S., and Suske, G. (1999) *Nucleic Acids Res.* **27,** 2991–3000
21. Suske, G. (1999) *Gene* **238,** 291–300
22. Brandeis, M., Frank, D., Keshet, I., Siegfried, Z., Mendelsohn, M., Nemes, A., Temper, V., Razin, A., and Cedar, H. (1994) *Nature* **371,** 435–438
23. Ainbinder, E., Revach, M., Wolstein, O., Moshonov, S., Diamant, N., and Dikstein, R. (2002) *Mol. Cell. Biol.* **22,** 6354–6362
24. Suzuki, T., Kimura, A., Nagai, R., and Horikoshi, M. (2000) *Genes Cells* **5,** 29–41
25. Ryu, S., Zhou, S., Ladurner, A. G., and Tjian, R. (1999) *Nature* **397,** 446–450
26. Kadam, S., and Emerson, B. M. (2003) *Mol. Cell* **11,** 377–389
27. Doetzlhofer, A., Rotheneder, H., Lagger, G., Koranda, M., Kurtev, V., Brosch, G., Wintersberger, E., and Seiser, C. (1999) *Mol. Cell. Biol.* **19,** 5504–5511
28. Won, J., Yim, J., and Kim, T. K. (2002) *J. Biol. Chem.* **277,** 38230–38238
29. Suske, G. (2000) *Methods Mol. Biol.* **130,** 175–187
30. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) *Nucleic Acids Res.* **17,** 6419
31. Hagen, G., Muller, S., Beato, M., and Suske, G. (1994) *EMBO J.* **13,** 3843–3851
32. Takahashi, Y., Rayman, J. B., and Dynlacht, B. D. (2000) *Genes Dev.* **14,** 804–816
33. Morrison, A. J., Sardet, C., and Herrera, R. E. (2002) *Mol. Cell. Biol.* **22,** 856–865
34. Sadee, W., Yu, V. C., Richards, M. L., Preis, P. N., Schwab, M. R., Brodsky, F. M., and Biedler, J. L. (1987) *Cancer Res.* **47,** 5207–5212
35. Lang, S. E., McMahon, S. B., Cole, M. D., and Hearing, P. (2001) *J. Biol. Chem.* **276,** 32627–32634
36. McMahon, S. B., Van Buskirk, H. A., Dugan, K. A., Copeland, T. D., and Cole, M. D. (1998) *Cell* **94,** 363–374
37. Ait-Si-Ali, S., Polesskaya, A., Filleur, S., Ferreira, R., Duquet, A., Robin, P., Vervish, A., Trouche, D., Cabon, F., and Harel-Bellan, A. (2000) *Oncogene* **19,** 2430–2437
38. Cosma, M. P., Tanaka, T., and Nasmyth, K. (1999) *Cell* **97,** 299–311
39. Muller, H., Bracken, A. P., Vernell, R., Moroni, M. C., Christians, F., Grassilli, E., Prosperini, E., Vigo, E., Oliner, J. D., and Helin, K. (2001) *Genes Dev.* **15,** 267–285
40. El-Osta, A., Kantharidis, P., Zalcberg, J. R., and Wolffe, A. P. (2002) *Mol. Cell. Biol.* **22,** 1844–1857
41. Suzuki, H., Gabrielson, E., Chen, W., Anbazhagan, R., van Engeland, M., Weijenberg, M. P., Herman, J. G., and Baylin, S. B. (2002) *Nat. Genet.* **31,** 141–149
42. Vigo, E., Muller, H., Prosperini, E., Hateboer, G., Cartwright, P., Moroni, M. C., and Helin, K. (1999) *Mol. Cell. Biol.* **19,** 6379–6395
43. Di Fiore, B., Palena, A., Felsani, A., Palitti, F., Caruso, M., and Lavia, P. (1999) *Nucleic Acids Res.* **27,** 2852–2859
44. Campanero, M. R., Armstrong, M. I., and Flemington, E. K. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 6481–6486
45. Bouchard, C., Dittrich, O., Kiermaier, A., Dohmann, K., Menkel, A., Eilers, M., and Luscher, B. (2001) *Genes Dev.* **15,** 2042–2047
46. Watanabe, G., Albanese, C., Lee, R. J., Reutens, A., Vairo, G., Henglein, B., and Pestell, R. G. (1998) *Mol. Cell. Biol.* **18,** 3212–3222
47. Chang, Y. C., Illenye, S., and Heintz, N. H. (2001) *Mol. Cell. Biol.* **21,** 1121–1131
48. van Ginkel, P. R., Hsiao, K. M., Schjerven, H., and Farnham, P. J. (1997) *J. Biol. Chem.* **272,** 18367–18374
49. Lin, S. Y., Black, A. R., Kostic, D., Pajovic, S., Hoover, C. N., and Azizkhan, J. C. (1996) *Mol. Cell. Biol.* **16,** 1668–1675
50. Karlseder, J., Rotheneder, H., and Wintersberger, E. (1996) *Mol. Cell. Biol.* **16,** 1659–1667
51. Blau, J., Xiao, H., McCracken, S., O'Hare, P., Greenblatt, J., and Bentley, D. (1996) *Mol. Cell. Biol.* **16,** 2044–2055
52. Wade, P. A. (2001) *BioEssays* **23,** 1131–1137
53. Harrington, M. A., Jones, P. A., Imagawa, M., and Karin, M. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 2066–2070
54. Agalioti, T., Lomvardas, S., Parekh, B., Yie, J., Maniatis, T., and Thanos, D. (2000) *Cell* **103,** 667–678
55. Ross, R. A., Spengler, B. A., and Biedler, J. L. (1983) *J. Natl. Cancer Inst.* **71,** 741–747
56. Zimmerman, K., Legouy, E., Stewart, V., Depinho, R., and Alt, F. W. (1990) *Mol. Cell. Biol.* **10,** 2096–2103