THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 23, Issue of June 8, pp. 20482–20490, 2001

Printed in U.S.A.

The Chromatin Structure of the Dual c-*myc* Promoter P1/P2 Is Regulated by Separate Elements*

Received for publication, January 11, 2001, and in revised form, February 12, 2001
Published, JBC Papers in Press, February 20, 2001, DOI 10.1074/jbc.M100265200

Thomas Albert‡, Julie Wells§, Jens-Oliver Funk¶, Andrea Pullner, Eva-Elisabeth Raschke, Gertraud Stelzer‖, Michael Meisterernst‖, Peggy J. Farnham§, and Dirk Eick**

From the Institute of Clinical Molecular Biology and Tumor Genetics and ‖Department for Protein Chemistry, Research Centre for Environment and Health (GSF), Marchioninistrasse 25, D-81377 München, Germany and the §McArdle Laboratory for Cancer Research, University of Wisconsin Medical School, Madison, Wisconsin 53706

The proto-oncogene c-*myc* is transcribed from a dual promoter P1/P2, with transcription initiation sites 160 base pairs apart. Here we have studied the transcriptional activation of both promoters on chromatin templates. c-*myc* chromatin was reconstituted on stably transfected, episomal, Epstein-Barr virus-derived vectors in a B cell line. Episomal P1 and P2 promoters showed only basal activity but were strongly inducible by histone deacetylase inhibitors. The effect of promoter mutations on c-*myc* activity, chromatin structure, and E2F binding was studied. The ME1a1 binding site between P1 and P2 was required for the maintenance of an open chromatin configuration of the dual c-*myc* promoters. Mutation of this site strongly reduced the sensitivity of the core promoter region of P1/P2 to micrococcal nuclease and prevented binding of polymerase II (pol II) at the P2 promoter. In contrast, mutation of the P2 TATA box also abolished binding of pol II at the P2 promoter but did not affect the chromatin structure of the P1/P2 core promoter region. The E2F binding site adjacent to ME1a1 is required for repression of the P2 promoter but not the P1 promoter, likely by recruitment of histone deacetylase activity. Chromatin precipitation experiments with E2F-specific antibodies revealed binding of E2F-1, E2F-2, and E2F-4 to the E2F site of the c-*myc* promoter *in vivo* if the E2F site was intact. Taken together, the analyses support a model with a functional hierarchy for regulatory elements in the c-*myc* promoter region; binding of proteins to the ME1a1 site provides a nucleosome-free region of chromatin near the P2 start site, binding of E2F results in transcriptional repression without affecting polymerase recruitment, and the TATA box is required for polymerase recruitment.

The nucleosomal structure of promoter regions constitutes an essential regulatory mechanism of eukaryotic gene repression. Gene activation is accompanied by perturbations or alterations of the nucleosomal structure like remodeling and acetylation of chromatin (1, 2). A first critical step for activating a gene locus seems to be liberating the promoter region from histone-mediated repression (1, 2). The transcription complex seems not to be recruited to the promoter until positioned nucleosomes have been disrupted by the concerted action of invading chromatin-remodeling and *trans*-acting factors (1, 2). A large number of chromatin remodeling activities have been isolated and characterized biochemically. The properties of remodeled nucleosomes include increased accessibility of DNA to DNA-binding proteins throughout the nucleosome (3–5), rotational phasing of DNA on the histone octamer (6), reduction of the total length of DNA per nucleosome (7), octamer susceptibility to displacement *in trans* (8), nucleosome movement without disruption or *trans*-displacement of histone octamer (9), and other effects.

Chromatin remodeling activities transfer promoters into a configuration that is accessible for sequence-specific binding of transcription factors and the recruitment of the transcription machinery. After initiation, the transcriptional machinery requires further activation signals for processive transcription. An increasing number of genes, including heat-shock genes (10, 11), c-*fos* (12), immunoglobulin κ (13), and c-*myc* (14–16), has been shown to be regulated by promoter proximal pausing of RNA polymerase II (pol II).[1]

The c-*myc* gene is transcribed from the dual P1 and P2 promoters that are located 160 bp apart. Both promoters have been studied intensively in *in vitro* and transient transfection experiments as well as in *Xenopus* oocytes and transgenic mice (for reviews see Refs. 17–19). In normal cells, c-*myc* is transcribed predominantly from the P2 promoter. In addition to the TATA box, two further elements have been identified required for P2 activity: a ME1a1 site at position −40 bp and a E2F-binding site at −58 bp relative to the P2 transcription start (20–23). Mutation of either or both sites strongly reduced P2 activity in transient transfection assays. Stable transfection of c-*myc* promoter constructs in cell lines or transgenic mice consistently led to an inactivation of the dual c-*myc* promoter P1/P2 (24). Repression of c-*myc* was also observed after stably introducing the gene on episomal vectors in human B cell lines. The episomal c-*myc* established a chromatin structure indistinguishable to the chromosomal c-*myc* with identical positions for nucleosomes and DNase I-hypersensitive sites. Moreover, a

* This work was supported by Die Deutsche Forschungsgemeinschaft (SFB190) and Fonds der Chemischen Industrie. 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.

‡ Present address: Lab. for Physiological Chemistry and Centre for Biomedical Genetics, Utrecht University, P. O. Box 80042, 3508 TA Utrecht, The Netherlands.

¶ Present address: Lab. of Molecular Tumor Biology, Dept. of Dermatology, University of Erlangen, 91052 Erlangen, Germany.

** To whom correspondence should be addressed: Institute of Clinical Molecular Biology and Tumor Genetics, GSF Research Centre, Marchioninistr. 25, D-81377 München, Germany. Tel.: +49-89-7099512; Fax: +49-89-7099500; E-mail: eick@gsf.de.

[1] The abbreviations used are: pol, polymerase; bp, base pair(s); wt, wild type; PCR, polymerase chain reaction; Pipes, 1,4-piperazinediethanesulfonic acid; MNase, micrococcal nuclease; nt, nucleotide(s); SoB, sodium butyrate; MAZ, MYC-associated zinc finger protein; HSI, DNase I-hypersensitive site I; HDAC, histone deacetylase; Rb, retinoblastoma tumor suppressor.

paused pol II is detectable downstream of the P2 start site. Transcription from the episomal c-*myc* promoters was strongly inducible by inhibitors of histone deacetylases (16, 25–27).

In the present study we aimed at extending the aforementioned findings on c-*myc* regulation by mutagenizing promoter elements of the episomal genes. We deleted the P2 TATA box and mutated the ME1a1 and E2F site. We asked to what extent deletion of the TATA box abolishes binding and pausing of pol II at the P2 promoter, and if so, how does the absence of pol II affect the chromatin structure of the promoter? We further asked whether mutation of the ME1a1 and E2F sites affects c-*myc* expression at the same regulatory level. The results were quite unexpected and gave deeper insight into the architecture and regulation of the c-*myc* promoter *in vivo*.

MATERIALS AND METHODS

Cell Lines and Cell Culture—Cell lines were obtained by stable transfection of Raji cells with the DNA containing the 8.1-kilobase pair *Hin*dIII-*Eco*RI c-*myc* gene locus on the episomal, self-replicating Epstein-Barr virus-derived vector, pHEBOPL. Raji is an Epstein-Barr virus-positive Burkitt's lymphoma cell line with a t(8;14) translocation expressing a functional Rb protein. wt cells contained a construct with c-*myc* germline sequences. ΔP2TATA, E2Fmt, ME1a1mt, and ΔP1/P2TATA (Fig. 1) were constructed starting from previously described deletions and *Eco*RI scanner mutants (28), which were introduced into the wt construct by PCR technology. Cells were grown to a density of 1×10^6 cells/ml in 10% fetal calf serum, RPMI 1640 medium (Life Technologies, Inc.) supplemented with penicillin, streptomycin, and L-glutamine. Medium for the c-*myc* transfectants contained additionally 300 μg/ml hygromycin B. Treatment of the cells with sodium butyrate was for 16 h at a final concentration of 3 mM.

Preparation of Total Cellular RNA—Approximately 1×10^8 cells were washed with ice-cold phosphate-buffered saline and spun down at 1200 rpm for 10 min at 4 °C. The pellet was resuspended in 20 ml of a 4 M guanidinium isothiocyanate solution (Sigma) and sheared by drawing the suspension into a syringe and expelling it through a 23-gauge needle several times until the preparation was no longer viscous. RNA was pelleted through a CsCl cushion as described (29).

Preparation of Nuclei—Isolation of the nuclei was carried out as described (14). Briefly, cells were spun down at 1200 rpm for 10 min at 4 °C and washed twice with ice-cold phosphate-buffered saline, and the pellets of 1×10^8 cells were resuspended in 10 mM Tris/HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% (v/v) Nonidet P-40. After incubation on ice for 5 min, the lysate was spun down at 1500 rpm for 15 min at 4 °C. The pelleted nuclei were resuspended in storage buffer (50 mM Tris/HCl, pH 8.3, 40% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA) and immediately frozen in liquid nitrogen in portions of 100 μl corresponding to 2×10^7 nuclei.

Nuclease S1 Mapping—A single-stranded uniformly labeled DNA probe was prepared by primer extension of a M13 clone. Labeling of the probe and hybridization of labeled DNA fragments to RNA was carried out according to Berk and Sharp (30). Hybridization mixtures of 30 μl containing 1×10^5 cpm of the probe (specific activity 10^8 cpm/μg), 30 μg RNA, 90% (v/v) formamide, 400 mM NaCl, 40 mM Pipes, pH 6.4, and 1 mM EDTA were denatured at 90 °C for 5 min and immediately transferred to 56 °C. After at least 12 h the hybridization process was terminated by the addition of 180 μl of ice-cold stop buffer containing 250 mM NaCl, 30 mM sodium acetate, pH 4.2, 2 mM zinc acetate, 5% (v/v) glycerol, and 400 units of nuclease S1 (Roche Molecular Biochemicals). The samples were incubated at 25 °C for 1 h, extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v), and precipitated with ethanol. Protected DNA fragments were separated on 5% (w/v) polyacrylamide gels.

Mapping of Nucleosomes by Micrococcal Nuclease (MNase)—Nuclei were isolated essentially as described above. 2×10^7 nuclei in 200 μl of buffer (30 mM Tris-HCl, pH 8.3, 150 mM KCl, 10 mM $CaCl_2$, 5 mM $MgCl_2$, 20% glycerol, 0.05 mM EDTA) were incubated for increasing periods of times with 3 units of MNase (Sigma) at room temperature. Chromatin was cut to various extents in MNase digestion kinetics. The reaction was stopped by the addition of 10 μl 0.5 M EDTA, and DNA was purified as described (26). For further analysis, DNA samples were chosen in which ~5–10% and 30–40% of nucleosomal spacers had been cut by MNase. In addition, nuclease-digested DNA was cut with either restriction endonuclease *Xba*I, *Acc*I, or *Hin*dIII in conditions recommended by the manufacturer (New England Biolabs). DNA fragments were separated in a 2% (w/v) agarose gel, denatured by alkali treatment, transferred to a nylon membrane (Hybond N+, Amersham Pharmacia Biotech), and hybridized with multi-prime labeled PCR probes as indicated. Hybridization probes A (nt 111–315), L (nt 1865–2065), and T (nt 2881–3085) were generated by PCR using specific c-*myc* primers and multi-prime labeled with [α-^{32}P]dCTP. Hybridization was carried out for 24 h in Church buffer (7% (w/v) SDS, 0.5 M sodium phosphate, 1 mM EDTA, pH 7.1) at 65 °C. Membranes were washed at room temperature with 1% SDS, 0.1× SSC (0.15 M NaCl, 15 mM sodium citrate, 1 mM EDTA, pH 7.5) and at 50 °C to achieve higher stringency. After drying, filters were exposed to Kodak X-Omat AR film at −80 °C with intensifying screens.

Nuclear Run-on Assay—Isolation of nuclei, purification, and hybridization of labeled RNA to membrane-bound oligonucleotides and the washing procedure of membranes including the digestion of single-stranded RNA with RNase A have been described in detail elsewhere (14, 53). Briefly, 100 μl corresponding to 2×10^7 isolated nuclei in storage buffer (50 mM Tris/HCl, pH 8.3, 40% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA) were thawed on ice and subsequently incubated with 100 μl of reaction buffer (10 mM Tris/HCl, pH 8.0, 5 mM $MgCl_2$, 300 mM KCl, 0.5 mM ATP, GTP, and UTP, and 100 μCi of [α-^{32}P]CTP (800 Ci/mmol, Amersham Pharmacia Biotech)) for 15 min at 28 °C. Nuclear transcripts were isolated, and labeled RNA was hybridized to DNA oligonucleotides immobilized on a nylon membrane (Hybond+, Amersham Pharmacia Biotech) at 65°C for at least 48 h in 5 ml of Church buffer. After being washed and dried, the membranes were exposed to Kodak X-Omat AR film at −80 °C with intensifying screens. The intensities of the hybridization signals were determined with a BAS 1000 phosphorimaging system (Fuji) and calculated relatively to signals obtained with a homogeneously labeled RNA transcribed by T7 RNA polymerase in the presence of [α-^{32}P]CTP. Oligonucleotides complementary to the human antisense strand, 50 nt long each, were synthesized according to the sequence described by Gazin *et al*. (31) and have been described elsewhere (14).

Preparation of in Vitro Transcribed RNA by T7 RNA Polymerase—For production of a uniformly labeled c-*myc* RNA, DNA fragments encompassing the c-*myc* region from positions 2328 to 2880 and from positions 1878 to 2638 were generated by PCR. PCR fragments were reamplified with primers carrying the T7 RNA polymerase promoter for *in vitro* transcription by T7 RNA polymerase (Roche Molecular Biochemicals). *In vitro* transcription was done essentially as recommended by the manufacturer in the presence of [α-^{32}P]CTP. Full-length transcripts were isolated by preparative polyacrylamide gel electrophoresis and used for hybridization to DNA oligonucleotides as described above.

In Vitro Transcription—Jurkat cells were grown to a density of 3.5×10^5/ml and harvested. Cells were resuspended in 4 cell volumes of buffer A (10 mM Tris-HCl, pH 7.3 (room temperature), 1.5 mM MgCl, 10 mM KCl), incubated for 10 min on ice, and spun down at 2000 rpm in a Heraeus Varifuge 3.0R. Cell pellets were resuspended in 2 volumes of buffer A and homogenized 10 times using a glass Dounce homogenizer with a type B pestle. The nuclei were spun down for 15 min at 3000 rpm at 4 °C, resuspended in 1 volume of buffer C (20 mM Tris-HCl pH 7.3 (room temperature), 50% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA) and homogenized 10 times in a Dounce homogenizer using a B pestle. Nuclei were extracted for 60 min at 4 °C and centrifuged for 30 min at 18,000 rpm in a SS34 rotor. The extract was dialyzed against buffer C (20 mM Tris-HCl pH 7.3 (room temperature), 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 5 mM dithiothreitol). For *in vitro* transcription reactions, 1.5 μg of the indicated DNA reporters were transcribed in the presence of 4 mM $MgCl_2$, 25 mM Hepes, pH 8.2, 5 mM dithiothreitol, 200 ng/μl bovine serum albumin (Roche Molecular Biochemicals), 1 mM phenylmethylsulfonyl fluoride, 100 μM ATP, GTP, and UTP, respectively, 5 μM CTP, and 50 μCi of [α-^{32}P]CTP (Amersham Pharmacia Biotech, 3000 Ci/mmol) in a reaction volume of 100 μl and otherwise under standard conditions (54). If indicated, α-amanitin was added to a final concentration of 0.5 μM. Nuclear extracts of Jurkat cells (50 μl or 250 μg OF nuclear extract) or a partially purified transcription system served as a source for general transcription factors and ubiquitous activators. To obtain the latter, nuclear extracts were loaded on phosphocellulose (P11 at 10 mg/ml beads) and step-eluted with 300, 500, and 850 mM KCl in buffer C. The P11 system consisted of 15 μg of the fraction eluting at 850 mM KCl and 40 μg of the fraction eluting at 500 mM KCl. Reactions were incubated for 1 h at 28 °C and processed as described below.

Formaldehyde Cross-linking and Immunoprecipitation—Cells were formaldehyde-cross-linked and immunoprecipitated as previously described (32). Cells were swelled in RSB buffer (3 mM $MgCl_2$, 10 mM NaCl, 10 mM Tris-HCl (pH 7.4), and 0.1% ICEPAL CA-330 (Sigma))

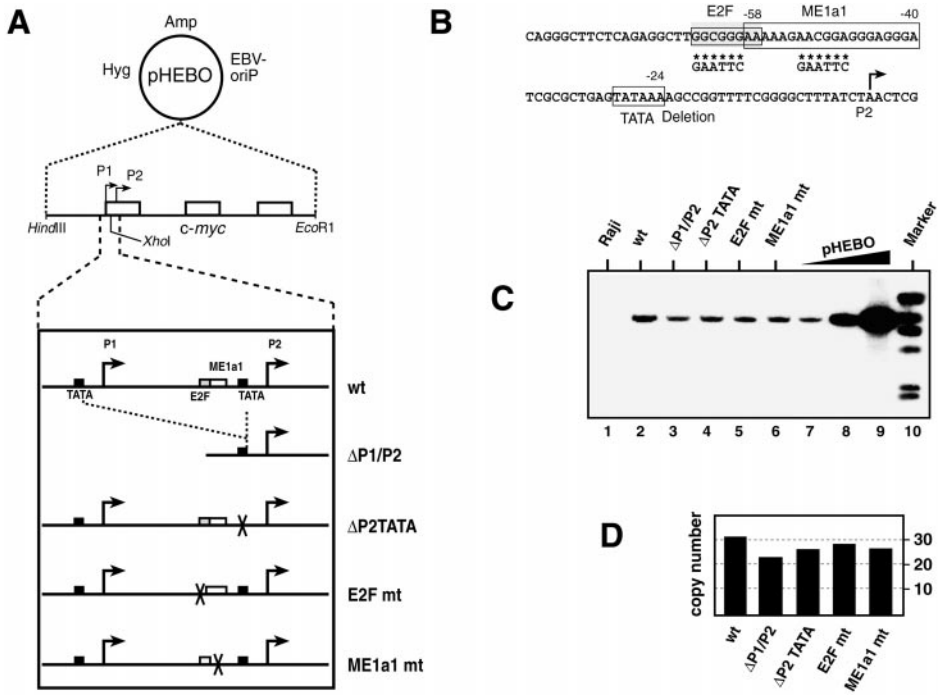

FIG. 1. **Episomal c-*myc* genes in stably transfected B cell lines.** *A*, episomal pHEBO-derived c-*myc* constructs used for stable transfections. Open boxes correspond to c-*myc* exons 1 to 3. All constructs carry the origin for episomal replication of the Epstein-Barr virus (*EBV-oriP*), the ampicillin resistance gene (*amp*), and the gene for hygromycin resistance (*Hyg*). Constructs contained the c-*myc* wild type promoter (*wt*), a deletion from the TATA box of the P1 promoter up to the TATA box of the P2 promoter (Δ*P1/P2*), a deletion of the TATA box of the P2 promoter (Δ*P2TATA*), an E2F site mutation, or an ME1a1 site mutation. *B*, detailed description of mutations. *C*, Southern blot analysis of episomal constructs in transfectants. *D*, determination of copy numbers.

instead of the reported buffer, in order to isolate cross-linked nuclei. Antibodies against E2F and Ets proteins, E2F-1 (sc-193), E2F-2 (sc-633), E2F-3 (sc-879), E2F-4 (sc-866), E2F-5 (a mixture of sc-1083 and sc-999) and Ets 1/2 (sc-112), were purchased from Santa Cruz Biotechnology. As controls, we included a reaction lacking primary antibody (No Ab) and a reaction that lacked chromatin (Mock). Each of the antibodies was shown by Western blot analysis to detect its cognate protein in NIH3T3 nuclear extracts (data not shown). Antibody-protein-DNA complexes were isolated by immunoprecipitation with blocked protein A-positive Staph A cells. Following extensive washing, bound DNA fragments were eluted and analyzed by subsequent PCR.

PCR Analysis—Immunoprecipitates were dissolved in 30 μl of water except for input samples, which were diluted in 100 μl and then further diluted 1:100. Each reaction contained 3 ml of immunoprecipitated chromatin, 1× *Taq* reaction buffer (Promega), 1.5 mM $MgCl_2$, 50 ng of each primer, 1.7 units of *Taq* polymerase (Promega), 200 μM each dNTP (Roche Molecular Biochemicals), and 1 M betaine (Sigma) in a final reaction volume of 20 μl. PCR reactions were amplified for 1 cycle of 95 °C for 5 min, annealing temperature of the primers for 5 min, 72 °C for 3 min, and 27 cycles of 95 °C for 1 min, annealing temperature of the primers for 2 min, 72 °C for 1.5 min. PCR products were separated by electrophoresis through a 1.5% agarose gel and visualized by ethidium bromide intercalation. The resulting PCR products were quantitated using ImageQuant Mac version 1.2 (Molecular Dynamics). Binding to the c-*myc* promoter episome in wt cells was analyzed using primers Myc-2411 and Myc-2857, in E2Fmt cells using primers Myc-E2Fmt and Myc-2857, and in ME1a1mt cells using primers Myc-ME1a1mt and Myc-2857. The sequences of the primers used are as follows: Myc-2411, 5′-GGCTTCTCAGAGGCTTGGCGGG-3′; Myc-2857, 5′-TCCAGCGTCTAAGCAGCTGCAA-3′; Myc-E2Fmt, 5′-GGCTTCTCAGAGGCTTGAATTC-3′; Myc-ME1a1, 5′-GCTTGGCGGGAAAAAGGAATTC-3′.

RESULTS

Activity of c-myc Promoter Mutants—We have previously shown that the episomal c-*myc* promoters P1 and P2 are repressed in stably transfected Raji cells. Although repression of the P1 promoter occurs at the level of initiation, the P2 promoter is repressed by promoter-proximal pausing of pol II (25). Transcription from episomal P1 and P2 promoters is strongly inducible by inhibitors of deacetylases (16, 27). In an extension of this work, we studied episomal c-*myc* promoter mutants. The E2F binding site at 58 bp and the ME1a1 site at 40 bp upstream of P2 were mutated by introducing *Eco*RI recognition sites (E2Fmt, ME1a1mt); the TATA box at 24 bp upstream of P2 was deleted (ΔP2TATA) (Fig. 1,*A* and *B*). As a fourth mutant the sequence between the TATA box of P1 and the TATA box of the P2 promoter was deleted leaving the TATA box of P1 intact (Δ*P1/P2*, Fig. 1*A*).

Expression of the wild type promoter and mutants were studied in the human B cell line Raji, stably transfected with DNA constructs, which contain an 8-kilobase pair fragment of the human c-*myc* gene together with the Epstein-Barr virus origin of replication (*oriP*), to allow episomal propagation in Epstein-Barr virus-positive cells (Fig. 1*A*). To prevent the formation of a functional c-Myc protein from the transfected constructs, a frameshift mutation was inserted into the coding part of c-*myc* exon 2. Cell batches were selected in the presence of hygromycin for ~4 weeks. The wt cell line and the four mutant cell lines ΔP1/P2, ΔP2TATA, E2Fmt, and ME1a1mt carried ~25 copies of the respective constructs (Fig. 1, *C* and *D*).

Basal expression of the episomal c-*myc* P2 promoter was studied by nuclease S1 analysis (Fig. 2*A*). The S1 probe, the size of protected fragments, and a quantification of the results are shown in Fig. 2, *B* and *C*. In wt, ME1a1mt, and ΔP2TATA cells, only low levels of P2-specific RNA were detectable (Fig. 2*A*, *lanes 1*, *5*, and *7*). In E2Fmt cells, the levels of P2-specific RNA were increased ~4-fold (*lane 3*). The mutations affected the basal transcription from the P1 promoter to various extents. Although E2Fmt and ME1a1mt did not significantly affect P1 activity (Fig. 2*A*, *lanes 3* and *5*), ΔP2TATA showed strongly increased basal activity of the P1 promoter (*lane 7*). Basal activity was also significantly increased for the chimeric ΔP1/P2 (*lane 9*). In wt cells, the treatment with an inhibitor of histone deacetylases, sodium butyrate (SoB), strongly induced transcription from the P1 and P2 promoter (*lane 2*), whereas the endogenous P1t promoter, in which expression is driven by Ig enhancers on the translocated c-*myc* allele, was repressed (*lane 2*). The basis for repression of P1t is unclear but probably is linked to the Ig locus because transcription of the non-translocated Ig heavy chain μ-gene in Raji cells is repressed also by SoB (data not shown). SoB did not induce the P2 promoter in ME1a1mt and ΔP2TATA cells (*lanes 6* and 8), whereas expression of P2 in E2Fmt cells was increased up to the levels seen in wt cells (*lane 4*). SoB was also able to induce

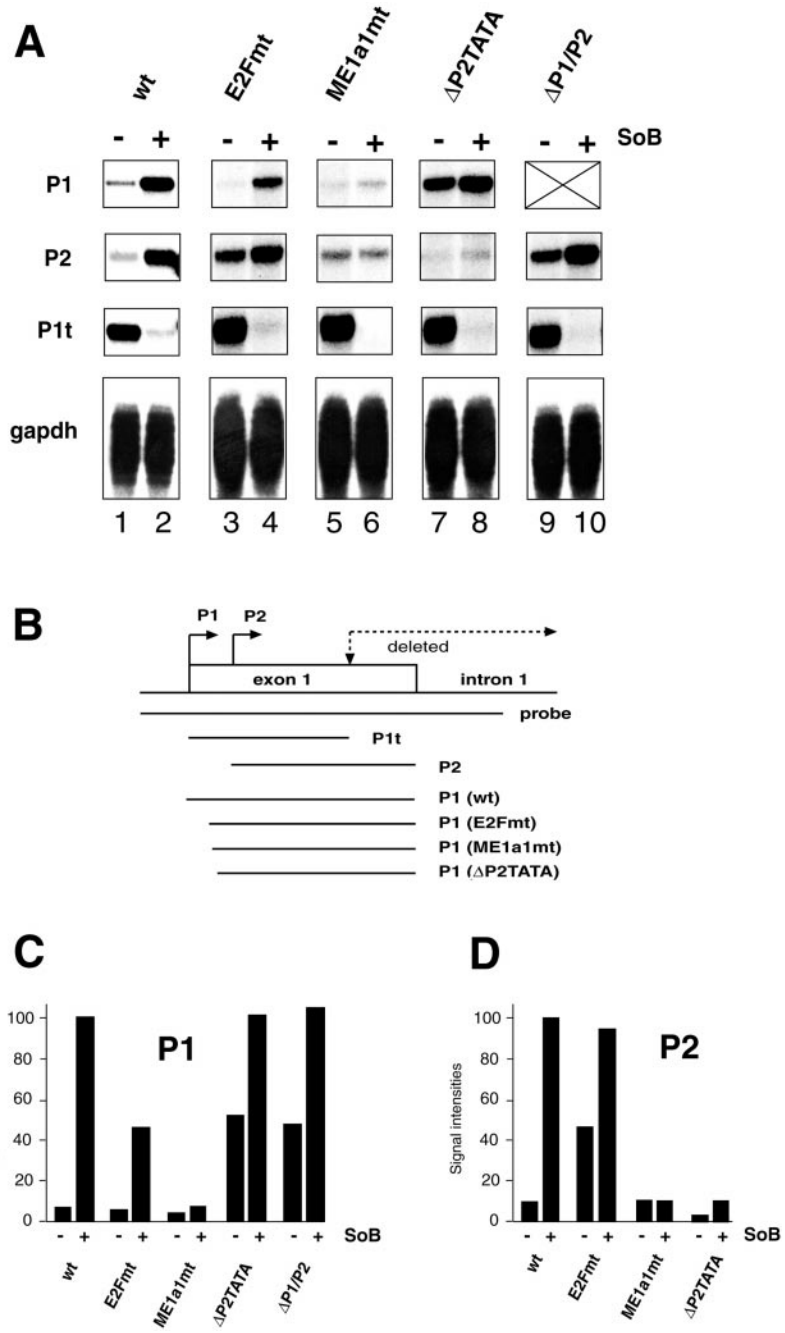

FIG. 2. **Analysis of RNAs expressed from stably transfected c-*myc* constructs in cell lines wt, E2Fmt, ME1a1, ΔP2TATA, and ΔP1/P2.** *A*, cells were cultivated in the absence (−) or presence (+) of 3 mM sodium butyrate (*SoB*) for 16 h. Total RNA was isolated, and c-*myc* RNA was studied by nuclease S1 analysis. The probe discriminated RNAs derived from the transfected constructs (*P1* and *P2*) and RNA from the active endogenous c-*myc* allele (*P1t*). A probe specific for glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) RNA served as internal control. *B*, description of the probe and of protected fragments. *C* and *D*, evalution of signal intensities obtained for the P1 (*C*) and P2 (*D*) promoters in the absence and presence of sodium butyrate, respectively.

the P1 promoter in E2Fmt cells (*lane 4*) but not in ME1a1mt cells (*lane 6*). In ΔP2TATA cells, the high basal levels of P1 RNA were further elevated by SoB up to levels as seen in wt cells (*lane 8*). Taken together, mutation of the TATA box and the ME1a1 site severely affected the inducibility of the P2 promoter. In addition, mutation of the ME1a1 site abolished the inducibility of P1. In contrast, mutation of the E2F site induced constitutive transcription from the P2 promoter but had only a minor effect on P1. For quantitative evaluation of signals, see Fig. 2, *C* and *D*.

Effect of Mutations on Binding and Pausing of pol II at the P2 Promoter—Expression of the c-*myc* P2 promoter is regulated mainly by pausing of pol II proximal to the start site (14, 15). To study whether mutations in the P2 promoter affect initiation at the P2 promoter or pausing of pol II, high resolution nuclear run-on experiments were performed. Hybridization of run-on RNAs to short oligonucleotides specific for c-*myc* exon 1 sequences allowed us to refine the resolution of the assay. Using this method we have shown previously that paused polymerases become transcriptionally activated in nuclear run-on reactions and transcribe a short piece of chromosomal and episomal c-*myc* RNA. The region transcribed after activation of these polymerases extends to ~100 bp downstream of the c-*myc* P2 promoter (16).

Nuclei of wt, E2Fmt, ME1a1mt, ΔP2TATA, and ΔP1/P2 cells were subjected to run-on reactions, and labeled RNAs were purified and hybridized to membrane-bound oligonucleotides A–L complementary to the entire c-*myc* exon 1 (Fig. 3*A*). The strong signals seen for oligonucleotides E and F are indicative of paused pol II, which becomes activated in the run-on reaction and transcribes a short stretch of RNA. We took the signals on oligonucleotides E and F as a measure of density of paused pol II. The signal strength for both oligonucleotides was high in wt cells (Fig. 3*A*, *lane 1*) but strongly reduced in ME1a1mt and ΔP2TATA cells (*lanes 3* and *4*). In contrast, the density of pol II at the P2 promoter in E2Fmt cells was in-

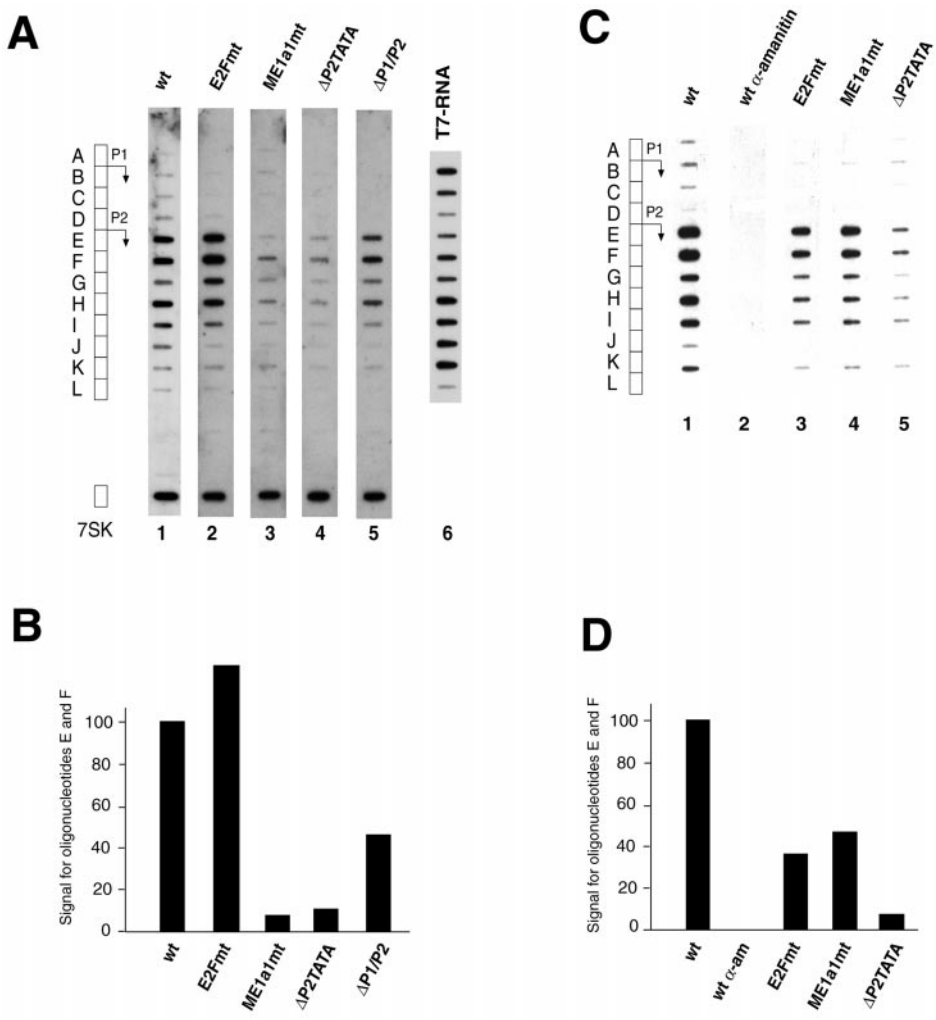

FIG. 3. **Pol II distribution within promoter-proximal sequences of c-*myc* genes.** *A*, the nuclei of wt, E2Fmt, ME1a1, ΔP2TATA, and ΔP1/P2 cells were isolated. Nuclear run-on reactions were performed in the presence of $\alpha[^{32}P]$CTP. Labeled RNAs were isolated and hybridized to membrane-fixed oligonucleotides. Oligonucleotides A–L correspond to the antisense strand of c-*myc* exon 1 with the promoters P1 and P2. Signals obtained with uniformly labeled c-*myc* RNA transcribed *in vitro* by T7 RNA polymerase (*T7-RNA*) served as a control for hybridization efficiency of the different oligonucleotides. The 7SK oligonucleotide served as the pol III transcription probe. *B*, run-on transcription signals were measured with a phosphorimager BAS 1000 system (Fuji). The transcriptional activities for oligonucleotides E and F of one representative experiment were determined relative to the corresponding signals in wt cells. *C*, constructs carrying wt c-*myc* and the mutants E2Fmt, ME1a1, and ΔP2TATA were subjected to an *in vitro* transcription experiment in the presence of [α-^{32}P]CTP. *D*, labeled RNAs were hybridized to oligonucleotides, and intensities of signals were evaluated as described above.

creased compared with wt cells (*lane 2*). Although the P1 wt promoter showed almost no pausing (Fig. 3*A*, *lane 1*, oligonucleotides B and C), significant pausing was seen at the chimeric ΔP1/P2 promoter (*lane 5*). All signals were calculated relatively to the signal obtained for the 7SK gene (Fig. 3*B*). These results support the hypothesis that the TATA box and the ME1a1 binding site are positive regulatory elements.

The E2F and ME1a1 sites in the c-*myc* promoter have been reported previously to be positive elements for the activity of the P2 promoter in transient transfection and in *in vitro* transcription experiments (20). We tested the activity of the mutant c-*myc* promoters in nuclear extracts from Jurkat cells (see "Materials and Methods"). Labeled transcripts were hybridized to c-*myc* exon 1 antisense oligonucleotides. A plasmid with the wt P2 promoter was able to produce large amounts of transcripts in nuclear extracts. However, similarly to nuclei, most of these transcripts were not full size, indicating that transcription was paused or prematurely terminated on the wt construct (Fig. 3*C*, *lane 1*). Transcription in extracts was entirely sensitive to α-amanitin (*lane 2*), indicating the complete dependence of c-*myc* transcription in these extracts on pol II. The mutant constructs E2Fmt and ME1a1mt showed 2.5- and 2-fold reduced transcriptional activity compared with the wt construct (Fig. 3*C*, *lanes 3* and *4*); the activity of ΔP2TATA was reduced >10-fold (*lane 5*). Taken together, a striking discrepancy for the transcriptional activity of E2Fmt and ME1a1mt is observed in nuclei *versus in vitro* transcription experiments. Both mutants have similar activity in *in vitro* transcription experiments. However, in the context of chromatin in isolated nuclei, they display striking differences; E2Fmt is transcriptionally active and has high amounts of pol II bound, whereas ME1a1mt is transcriptionally inactive and is not bound by significant amounts of pol II.

The Mutants ME1a1mt and ΔP1/P2 Have an Altered Chromatin Structure—The observed differences of transcription experiments *in vitro* and in nuclei suggested that altered chromatin structures in c-*myc* mutants might affect the binding of pol II to the P2 promoter and inducibility of the P1/P2 promoters by SoB. Therefore, we studied the nucleosomal structure of the P1/P2 promoter region after MNase digestion. The nuclei from the various cell lines were treated with MNase for increasing periods of time, and the DNA was purified and separated by gel electrophoresis. The resulting DNA fragments displayed the characteristic nucleosomal ladder (Fig. 4*L*). A digest of 20 min produced mostly mono-, di-, and trinucleosomal fragments (Fig. 4*L*, *lane 6*) with decreased fragment length compared with fragments from a 3-min digest (*lane 5*). The same pattern emerged when DNA was cut, in addition to MNase, with a restriction endonuclease (*lanes 2* and 3). DNA fragments were hybridized with the radioactively labeled probe T homologous to sequences downstream of the P2 promoter region. Using this probe, more than five nucleosomes were detected in all five cell lines (Fig. 4, *A*, *B*, *D*, and *E*, *lane 5*). This pattern was restricted to five nucleosomes if DNA was cut with *Xba*I (Fig. 4, *A–E*, *lane 2*), indicating that the region 70–100 bp downstream of the P2 cap site was hypersensitive to MNase in cell lines wt, E2Fmt, ΔP2TATA, and ΔP1/P2. This region was less sensitive to MNase digestion in ME1a1mt cells (Fig. 4*C*,

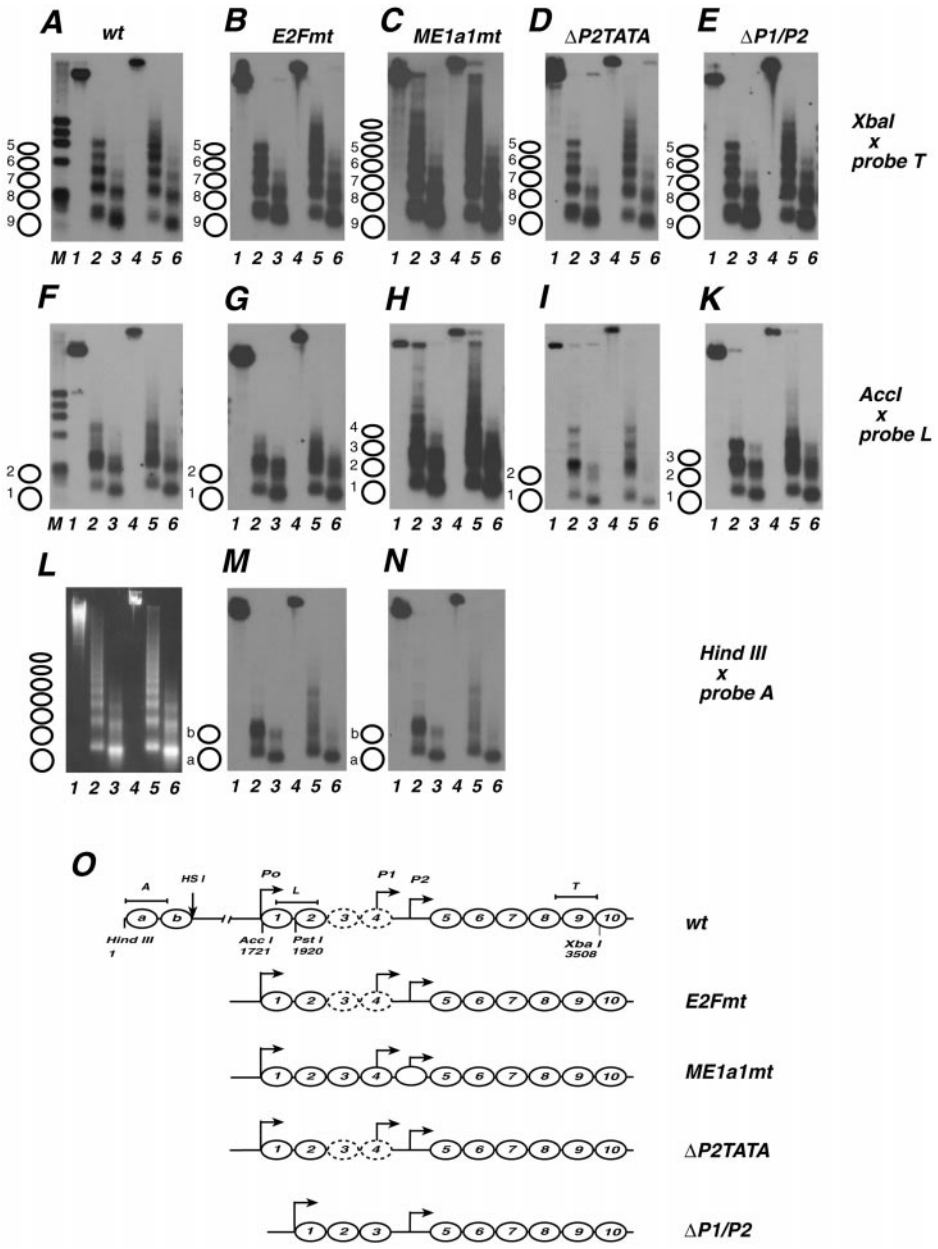

FIG. 4. **Nucleosomal patterns upstream and downstream of the c-*myc* P2 promoter on episomal constructs.** *A–K, M,* and *N*, Southern blot analysis of MNase-digested DNA of wt, E2Fmt, ME1a1, DP2TATA, and ΔP1/P2 cell lines. *L*, ethidium bromide staining of nucleosomal DNA corresponding to the Southern blot shown in *N*. Nuclei were isolated and incubated with MNase for different periods of time. Subsequently, DNA was purified and subjected to Southern analysis either uncut (*lanes 4–6*) or cut with restriction endonucleases *Acc*I, *Xba*I, and *Hin*dIII as indicated (*lanes 1–3*). The PCR fragments A, L, and T were used as hybridization probes for nucleosomal patterns upstream (probes A and L) or downstream (probe T) of the c-*myc* P2 promoter. *Lanes 1* and *4*, DNA treated with no MNase; *lanes 2* and *5*, DNA treated with MNase for 3 min; *lanes 3* and *6*, DNA treated with MNase for 20 min; *M*, labeled fX174 DNA-*Hae*III digest (New England Biolabs) serving as the molecular weight standard. Nucleosomes detected after restriction enzyme digest are shown schematically on the *left side* of each *panel*. *O*, scheme depicting the relative locations of nucleosomes and probes A, L, and T within the c-*myc* promoter region. The positions of restriction endonuclease recognition sites refer to the previously published c-*myc* sequence (31). Nucleosomes are numbered from 1 to 10; *stippled* nucleosomes are not detectable in all cell lines.

lane 2). To exclude the possibility that MNase might have cut chromatin in the nuclei of ME1a1mt cells to a lesser extent than in the nuclei of other cells, we compared the hypersensitivity of HSI (DNase I-hypersensitive site I) to MNase in nuclei of E2Fmt and ME1a1 cells (Fig. 4, *M* and *N*). DNA was cut with *Hin*dIII and hybridized with probe A. Two nucleosomes, a and b, were detectable, indicating that HSI showed a similar sensitivity to MNase in the nuclei of both cell lines. Partial digestion of naked cellular DNA with MNase did not produce the pattern of a nucleosomal ladder after hybridization with probe T (data not shown).

Differences in c-*myc* chromatin could be confirmed by hybridization with probe L upstream of P2. Probe L detected two nucleosomes downstream of the *Acc*I site (nucleosomes 1 and 2) in the chromatin of wt and E2Fmt cells (Fig. 4, *F* and *G*, *lane 2*). A hypersensitive site beyond nucleosome 2 led to an interruption of the ladder. The chromatin of ME1a1mt cells was less sensitive to MNase, and additional nucleosomes (nucleosomes 3 and 4) were detectable (Fig. 4*H*, *lane 2*). A significant change was also observed for the chromatin of ΔP1/P2 cells. These cells clearly established the nucleosome 3 (Fig. 4*K*, *lane 2*), which was only marginally detectable in chromatin of wt cells. If DNA was cut with the restriction enzyme *Pst*I (Fig. 4*O*), the nucleosomal ladders seen in *lanes 2* and *3* (Fig. 4, *F–K*) were shortened by exactly one nucleosome (data not shown). Taken together, clear differences were detectable in the c-*myc* chromatin of ME1a1mt and ΔP1/P2 cells compared with wt cells. The differences are depicted schematically in Fig. 4*O*.

Occupancy of the E2F Site at the c-myc Promoter in Vivo—E2F activity consists of a heterodimer containing one of six factors (E2F-1, E2F-2, E2F-3, E2F-4, E2F-5, and E2F-6) that pairs with a second subunit (DP-1 or DP-2) (for review see Ref. 33). The transcriptional activation potential of E2F is counterbalanced by pocket proteins, which tightly associate with E2F in a cell cycle-dependent manner (34, 35) and can recruit histone deacetylase activity to E2F sites (36). Because mutation of the E2F binding site resulted in such striking differences in c-*myc* P2 promoter activity *in vitro* and in living cells, we wanted to determine which members of the E2F factor family bind to the c-*myc* P2 promoter *in vivo*. To this end, we employed chromatin immunoprecipitation experiments to study transcription factor binding to the P2 upstream regulatory region in

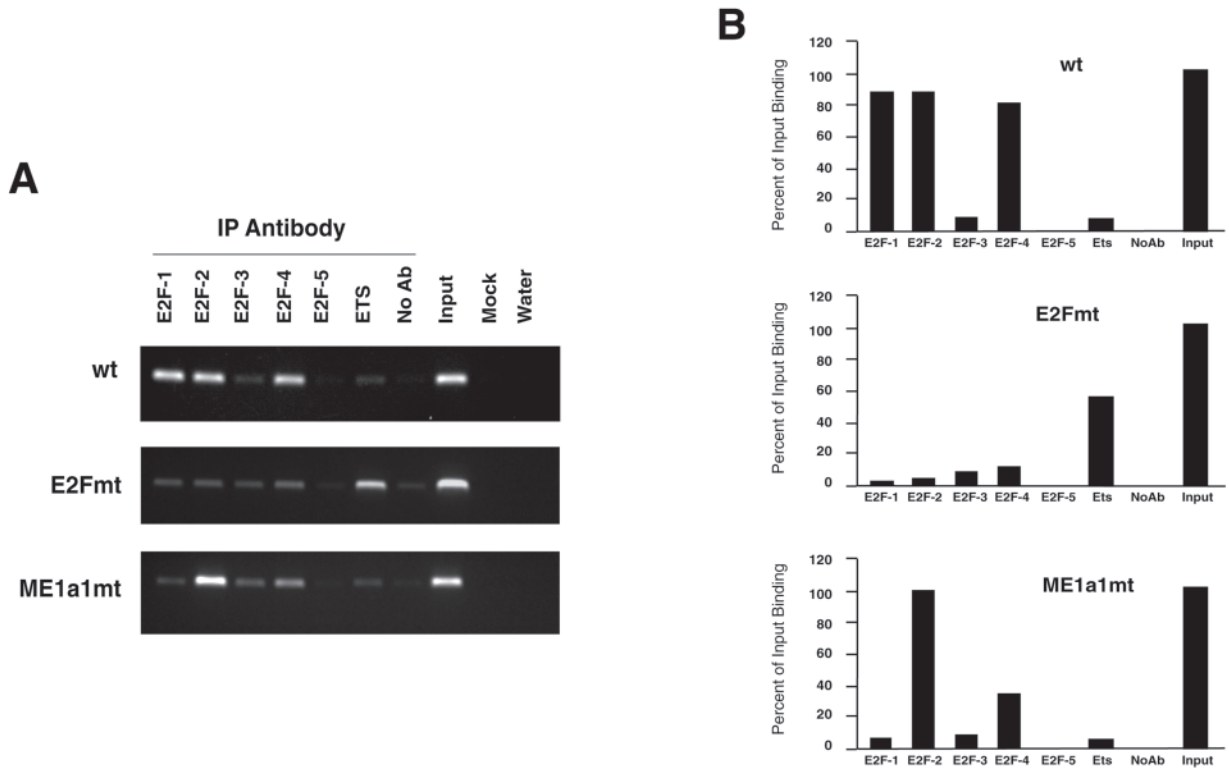

FIG. 5. **E2F-1, E2F-2, and E2F-4 bind to the c-*myc* promoter *in vivo*.** *A*, formaldehyde-cross-linked chromatin was prepared from wt, E2Fmt, and ME1a1mt cells, sonicated, and immunoprecipitated (*IP*) with specific antibodies as indicated. Immunoprecipitates from each antibody were aliquotted and subsequently analyzed by PCR with primers specific for the c-*myc* promoter. To verify that at each time point an equivalent amount of chromatin was used in the immunoprecipitations, a sample representing 0.02% of the total input chromatin (*Input*) was included in the PCR reactions. *No Ab*, no antibody. *B*, quantification of PCR results.

Raji cells (32, 35). Cross-linked chromatin from equivalent numbers of cells was immunoprecipitated using antibodies against E2F-1, E2F-2, E2F-3, E2F-4, and E2F-5. After immunoprecipitation and reversal of the cross-links, enrichment of the endogenous c-*myc*-promoter fragment in each sample was monitored by PCR amplification using gene specific primers. As shown in Fig. 5, the pattern of E2F binding differed on the c-*myc* wt promoter and the ME1a1mt and E2Fmt promoters. Anti-E2F-1, E2F-2, and E2F-4 immunoprecipitates from wt chromatin contain high levels of c-*myc*-promoter fragment. In contrast, the E2F-3- and E2F-5-specific antibodies failed to immunoprecipitate significant amounts of c-*myc* promoter fragment. Anti-E2F-1, E2F-2, and E2F-4 failed to immunoprecipitate significant amounts of the c-*myc* promoter fragment from cross-linked E2Fmt cells, indicating that E2F-1, E2F-2, and E2F-4 bind to the E2F site of the episomal c-*myc* promoter *in vivo*. Interestingly, mutation of the E2F binding site correlated with increased binding of Ets to the c-*myc* promoter; this is in agreement with the finding of Roussel *et al.* (37) that the binding of E2F and Ets to the c-*myc* promoter excludes each other. The notion that members of the E2F family might control binding of Ets to the c-*myc* promoter deserves further analysis. Inversely, we found evidence that binding of E2Fs to the c-*myc* promoter is controlled by factor binding in the neighborhood. Mutation of the ME1a1 site affected binding of E2F-1 and E2F-4 to the c-*myc* promoter but apparently had no effect on binding of E2F-2. Because of the lack of specific antibodies, the factors binding to the ME1a1 site in Raji cells have not been identified. In summary, several members of the E2F family bind to the c-*myc* promoter *in vivo,* and their binding is probably influenced or even specified by factors binding adjacent to them.

DISCUSSION

Pol II Binding to the c-myc P2 Promoter Can Be Dissected from Chromatin Opening—Previous studies have shown that the c-*myc* promoter region showed an open chromatin configuration if the gene was repressed. In addition, the repressed c-*myc* harbored a paused pol II proximal to P2 (16, 26–27). This observation raised the question of whether pol II itself, or pol II-associated factors, might be essential to inducing and keeping the open promoter configuration. The mutants analyzed in this study allowed us to answer this question. The ΔP2TATA mutant abolished initiation of pol II, and pausing of pol II was greatly reduced, as revealed by nuclease S1 and run-on experiments. However, the chromatin of this mutant still displayed hypersensitivity to MNase in the P1/P2 promoter region indicating that opening of c-*myc* in the promoter region is not linked to recruitment of pol II to the P2 promoter. Other elements in the promoter region, as described in the discussion of the ME1a1 site below, are critical for chromatin opening. The ΔP2TATA mutant also sheds light on the complexity of the dual c-*myc* promoters. Unexpectedly, the inhibition of pol II binding to the P2 promoter activates the P1 promoter, suggesting that P2 is a negative regulator of P1 transcription. We postulate a mechanism associated with the TATA box of P2 that negatively regulates the activity of P1. This idea has already been discussed for Burkitt lymphoma cells with t(8;14) translocations. In these cells c-*myc* is under the control of immunoglobulin enhancers, which constitutively activate the paused pol II at the P2 promoter. In the context of the dual promoter, liberation of the P2 pause site appears to be a prerequisite for P1 activation by immunoglobulin enhancers (25). In wt cells, the episomal P1 is strongly inducible by SoB. Because the activation

of P1 in ΔP2TATA cells is not increased further by SoB, but rather replaces the action of SoB, P2 may regulate the P1 activity by a mechanism involving acetylation.

ME1a1 Site Is Required for Chromatin Remodeling—Mutation of the ME1a1 site had the most severe effect on the activity of the P1/P2 promoter. This mutation abolished inducibility of the P1 as well as the P2 promoter. Consistently, paused pol II was no longer detectable at P2. Because ME1a1mt cells showed an altered nucleosomal structure for the P1/P2 promoter region compared with wt, it is likely that the ME1a1 binding factors are required for the opening of the c-*myc* promoter region. So far, two binding factors for the ME1a1 site have been described. MAZ (MYC-associated zinc finger protein) is a ubiquitously expressed protein able to activate c-*myc* reporter constructs in transient transfection assays (21). MAZ is essential for the ME1a1-mediated expression of the c-*myc* gene during neuroectodermal differentiation in P19 cells (40). Binding of MAZ to the ME1a1 motif in the c-*myc* core promoter is likely also to play an important role in the control of developmental expression of the CD4 gene (41) and of CLC-K1 and CLC-K2, two kidney-specific CLC chloride channel genes (42). The second known ME1a1 binding factor is hu-CUT, the human homolog of the *Drosophila* CUT homeodomain protein (43). hu-CUT represses a c-*myc* reporter gene in transient transfection assays. Our data suggest that the ME1a1 binding factors contribute to c-*myc* activation and that pol II cannot bind to the P2 promoter before c-*myc* chromatin has been opened by a ME1a1-dependent activity. This opening step cannot be substituted by inhibition of histone deacetylases, because SoB treatment was unable to induce transcription from the P1/P2 promoter in ME1a1mt cells. However, this does not rule out a function of ME1a1 binding factors in acetylation-dependent activation steps after opening of the promoter. Finally, the effect of the mutation of the ME1a1 site on c-*myc* chromatin is restricted locally and does not influence a HSI located 2 kilobase pairs upstream.

The E2F Site Negatively Regulates the P2 Promoter—The E2F site in the c-*myc* P2 promoter was first described as a positive element for c-*myc* expression. In fact, mutation of this site reduced P2 activity in transient transfection assays (20, 44) as well as in a pol II-dependent *in vitro* transcription assay (this work). Activation of a c-*myc* P2 reporter construct by E2F-1 overexpression is abrogated by Rb co-expression (44). This suggested that E2F sites can act as positive and negative elements for gene activity. Our results indicate that the binding of E2F factors is not required for activation of the P2 promoter. Inhibition of pocket protein recruitment to the E2F site appears to be sufficient for P2 activation. This finding is in line with observations in transgenic mice either overexpressing or having knocked-out E2F-1. Both types of transgenics establish tumors (45, 46). Although overexpression of E2F-1 probably titrates and thereby inactivates pocket proteins, loss of E2F-1 inhibits the recruitment of Rb to c-*myc* and other E2F-regulated promoters. Therefore, both overexpression and knockout of E2F-1 could lead to deregulation of c-*myc*. Can other members of the E2F family replace E2F-1 in c-*myc* repression? Chromatin immunoprecipitation experiments indicate that E2F-2 and E2F-4, in addition to E2F-1, bind to the c-*myc* promoter. Because E2F-2 is able to recruit Rb, E2F-1 and E2F-2 might be involved in the negative regulation of the episomal c-*myc* in Raji cells.

Similar to the c-*myc* P2 promoter, the cyclin E promoter is negatively regulated by Rb. The repressive role of Rb in cyclin E transcription has been mapped recently to its control of the acetylation status of a single nucleosome.[2] We have mapped the first nucleosomes next to the E2F site in the c-*myc* promoter at positions ~130 bp downstream (nucleosome 5) and ~450 bp upstream (nucleosome 2). Whether acetylation of these nucleosomes is controlled by the E2F site in the c-*myc* promoter remains to be demonstrated. However, other targets must be considered. Finally, in addition to HDAC activities recruited by the E2F site to the P2 promoter, additional HDAC activity probably contributes to P2 repression. Mutation of the E2F site leads to only ~50% promoter activation as compared with activation by SoB. A potential additional candidate is CTCF (CCCTC-binding protein), which has been shown to bind to the P2 core promoter and to recruit HDAC activity (47, 48).

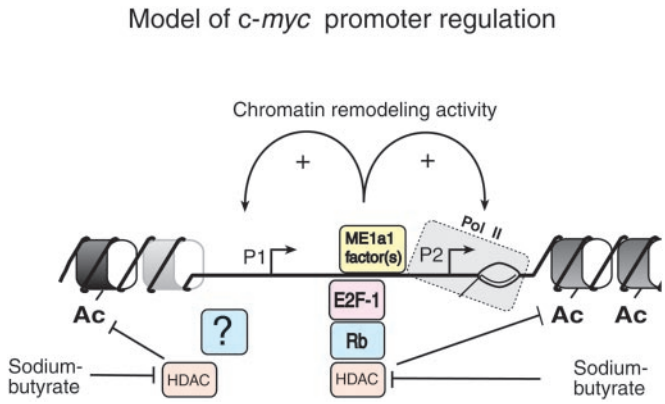

FIG. 6. **Model of c-*myc* regulation.** Although ME1a1 binding factors affect the global chromatin structure at the dual c-*myc* P1/P2 promoter, the E2F binding site is a negative site for P2 activity and probably recruits HDAC activity. P1 recruits HDAC activity by a yet unknown element.

Mutation of the E2F Site Does Not Activate the P1 Promoter—The E2F site is centered almost exactly between the transcription start sites of P1 and P2. Although mutation of this site strongly induced transcription from the P2 promoter, the adjacent P1 promoter was not activated. Interestingly, the P1 promoter is inducible by SoB to the same extent as the P2 promoter. From this observation we can draw the following conclusions: (i) the HDAC recruited to the E2F site is not able to repress transcription from the P1 promoter; and (ii) the activity of the P1 promoter is also controlled by a HDAC, but recruitment of this activity occurs by a separate element. The c-*myc* P1 promoter harbors several SP1 sites essential for its activity (49, 50). SP1 sites have recently been shown to be involved in PCAF (P300/CBP-associated factor)-mediated stimulation of transcription (51), as well as HDAC1-mediated repression of transcription (52).

Conclusions—This study has unraveled the hierarchical structures in the regulation of the dual c-*myc* promoters P1 and P2 (Fig. 6). Both promoters are under the common control of ME1a1 binding factors. This site controls the global chromatin structure in the promoter region of a wt c-*myc* gene and thereby probably the accessibility of the transcriptional machinery to each promoter. Once the c-*myc* promoter region is brought into an open chromatin configuration, pol II can initiate at P2 and subsequently pauses downstream thereof. The c-*myc* promoter region contains one single E2F site. This site negatively controls P2 activity by recruitment of HDAC activity. P1 promoter activity is controlled by HDAC in an E2F-independent manner.

Acknowledgments—We thank T. Meulia, A. Krumm, and M. Groudine for providing c-*myc* mutants.

[2] A. J. Morrison and R. E. Herrera, personal communication.

REFERENCES

1. Kingston, R. E., and Narlikar, G. J. (1999) *Genes Dev.* **13,** 2339–2352
2. Peterson, C. L., and Workman, J. L. (2000) *Curr. Opin. Genet. Dev.* **10,** 187–192
3. Cote, J., Quinn, J., Workman, J. L., and Peterson, C. L. (1994) *Science* **265,** 53–60
4. Kwon, H., Imbalzano, A. N., Khavari, P. A., Kingston, R. E., and Green, M. R. (1994) *Nature* **370,** 477–481
5. Logie, C., and Peterson, C. L. (1997) *EMBO J.* **16,** 6772–6782
6. Cairns, B. R., Lorch, Y., Li, Y., Zhang, M., Lacomis, L., Erdjument-Bromage, H., Tempst, P., Du, J., Laurent, B., and Kornberg, R. D. (1996) *Cell* **87,** 1249–1260
7. Bazett-Jones, D. P., Cote, J., Landel, C. C., Peterson, C. L., and Workman, J. L. (1999) *Mol. Cell. Biol.* **19,** 1470–1478
8. Lorch, Y., Zhang, M., and Kornberg, R. D. (1999) *Cell* **96,** 389–392
9. Langst, G., Bonte, E. J., Corona, D. F., and Becker, P. B. (1999) *Cell* **97,** 843–852
10. Rougvie, A. E., and Lis, J. T. (1988) *Cell* **54,** 795–804
11. Brown, S. A., Imbalzano, A. N., and Kingston, R. E. (1996) *Genes Dev.* **10,** 1479–1490
12. Plet, A., Eick, D., and Blanchard, J. M. (1995) *Oncogene* **10,** 319–328
13. Raschke, E. E., Albert, T., and Eick, D. (1999) *J. Immunol.* **163,** 4375–4382
14. Strobl, L. J., and Eick, D. (1992) *EMBO J.* **11,** 3307–3314
15. Krumm, A., Meulia, T., Brunvand, M., and Groudine, M. (1992) *Genes Dev.* **6,** 2201–2213
16. Wolf, D. A., Strobl, L. J., Pullner, A., and Eick, D. (1995) *Nucleic Acids Res.* **23,** 3373–3379
17. Spencer, C. A., and Groudine, M. (1991) *Adv. Cancer Res.* **56,** 1–48
18. Marcu, K. B., Bossone, S. A., and Patel, A. J. (1992) *Annu. Rev. Biochem.* **61,** 809–860
19. Levens, D., Duncan, R. C., Tomonaga, T., Michelotti, G. A., Collins, I., Davis-Smyth, T., Zheng, T., and Michelotti, E. F. (1997) *Curr. Top. Microbiol. Immunol.* **224,** 33–46
20. Moberg, K. H., Logan, T. J., Tyndall, W. A., and Hall, D. J. (1992) *Oncogene* **7,** 411–421
21. Bossone, S. A., Asselin, C., Patel, A. J., and Marcu, K. B. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 7452–7456
22. Hamel, P. A., Gill, R. M., Phillips, R. A., and Gallie, B. L. (1992) *Mol. Cell. Biol.* **12,** 3431–3438
23. Facchini, L. M., Chen, S., Marhin, W. W., Lear, J. N., and Penn, L. Z. (1997) *Mol. Cell. Biol.* **17,** 100–114
24. Lavenu, A., Pistoi, S., Pournin, S., Babinet, C., and Morello, D. (1995) *Mol. Cell. Biol.* **15,** 4410–4419
25. Strobl, L. J., Kohlhuber, F., Mautner, J., Polack, A., and Eick, D. (1993) *Oncogene* **8,** 1437–1447
26. Pullner, A., Mautner, J., Albert, T., and Eick, D. (1996) *J. Biol. Chem.* **271,** 31452–31457
27. Albert, T., Mautner, J., Funk, J. O., Hortnagel, K., Pullner, A., and Eick, D. (1997) *Mol. Cell. Biol.* **17,** 4363–4371
28. Meulia, T., Krumm, A., Spencer, C., and Groudine, M. (1992) *Mol. Cell. Biol.* **12,** 4590–4600
29. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual,* 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
30. Berk, A. J., and Sharp, P. A. (1977) *Cell* **12,** 721–732
31. Gazin, C., Dupont de Dinechin, S., Hampe, A., Masson, J. M., Martin, P., Stehelin, D., and Galibert, F. (1984) *EMBO J.* **3,** 383–387
32. Boyd, K. E., Wells, J., Gutman, J., Bartley, S. M., and Farnham, P. J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 13887–13892
33. Dyson, N. (1998) *Genes Dev.* **12,** 2245–2262
34. Takahashi, Y., Rayman, J. B., and Dynlacht, B. D. (2000) *Genes Dev.* **14,** 804–816
35. Wells, J., Boyd, K. E., Fry, C. J., Bartley, S. M., and Farnham, P. J. (2000) *Mol. Cell. Biol.* **20,** 5797–5807
36. Brehm, A., Miska, E. A., McCance, D. J., Reid, J. L., Bannister, A. J., and Kouzarides, T. (1998) *Nature* **391,** 597–601
37. Roussel, M., Davis, J. N., Cleveland, J. L., Ghysdael, J., and Hiebert, S. W. (1994) *Oncogene* **9,** 405–415
38. Deleted in proof
39. Deleted in proof
40. Komatsu, M., Li, H. O., Tsutsui, H., Itakura, K., Matsumura, M., and Yokoyama, K. K. (1997) *Oncogene* **15,** 1123–1131
41. Duncan, D. D., Stupakoff, A., Hedrick, S. M., Marcu, K. B., and Siu, G. (1995) *Mol. Cell. Biol.* **15,** 3179–3186
42. Uchida, S., Tanaka, Y., Ito, H., Saitoh-Ohara, F., Inazawa, J., Yokoyama, K. K., Sasaki, S., and Marumo, F. (2000) *Mol. Cell. Biol.* **20,** 7319–7331
43. Dufort, D., and Nepveu, A. (1994) *Mol. Cell. Biol.* **14,** 4251–4257
44. Oswald, F., Lovec, H., Moroy, T., and Lipp, M. (1994) *Oncogene* **9,** 2029–2036
45. Singh, P., Wong, S. H., and Hong, W. (1994) *EMBO J.* **13,** 3329–3338
46. Yamasaki, L., Jacks, T., Bronson, R., Goillot, E., Harlow, E., and Dyson, N. J. (1996) *Cell* **85,** 537–548
47. Filippova, G. N., Fagerlie, S., Klenova, E. M., Myers, C., Dehner, Y., Goodwin, G., Neiman, P. E., Collins, S. J., and Lobanenkov, V. V. (1996) *Mol. Cell. Biol.* **16,** 2802–2813
48. Lutz, M., Burke, L. J., Barreto, G., Goeman, F., Greb, H., Arnold, R., Schultheiss, H., Brehm, A., Kouzarides, T., Lobanenkov, V., and Renkawitz, R. (2000) *Nucleic Acids Res.* **28,** 1707–1713
49. DesJardins, E., and Hay, N. (1993) *Mol. Cell. Biol.* **13,** 5710–5724
50. Geltinger, C., Hortnagel, K., and Polack, A. (1996) *Gene Expr.* **6,** 113–127
51. Krumm, A., Madisen, L., Yang, X. J., Goodman, R., Nakatani, Y., and Groudine, M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 13501–13506
52. Doetzlhofer, A., Rotheneder, H., Lagger, G., Koranda, M., Kurtev, V., Brosch, G., Wintersberger, E., and Seiser, C. (1999) *Mol. Cell. Biol.* **19,** 5504–5511
53. Eick, D., and Bornkamm, G. W. (1986) *Nucleic Acids Res.* **14,** 8331–8346
54. Xie, J., Collart, M., Lemaire, M., Stelzer, G., and Meisterernst, M. (2000) *EMBO J.* **19,** 672–682