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 RNA 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 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 from the chromosomal c-myc with identical positions for nucleosomes and DNase I-hypersensitive sites. Moreover, a

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; mt, nucleotide(s); SoB, sodium butyrate; MAZ, MYC-associated zinc finger protein; HS1, DNase I-hypersensitive site 1; HDAC, histone deacetylase; Rb, retinoblastoma tumor suppressor.
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premature 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 HindIII-EcoRI 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. P2TATA, E2Fmt, ME1a1mt, and AP1/P2TATA (Fig. 1) were constructed starting from previously described deletion and EcoRI scanner mutants (28), which were introduced into the wt construct by PCR technology. Cells were grown to a density of 1 × 10^7 nuclei in 200 ml of ice-cold saline and suspended at 1200 rpm for 10 min at 4 °C. The pellet was resuspended in 20 ml of a 4 mM NaCl, 3 mM MgCl2, and 0.5% (v/v) Nonidet P-40 buffer (buffer A) and homogenized 10 times using a glass Dounce homogenizer and a Heraeus Varifuge 3.0R. Cell pellets were resuspended in 2 volumes of buffer A (10 mM Tris-HCl, pH 7.3, 1.5 mM MgCl2, 10 mM KCl, 1 mM EDTA, pH 7.1) at 65 °C. Membranes were washed at room temperature with 1% SDS, 0.1× SSC (0.15 mM 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 MgCl2, 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 MgCl2, 300 mM KCl, 0.5 mM ATP, GTP, and UTP, and 100 μCi of [α-32P]CTP (800 Ci/mmole, 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-N, Amersham Pharmacia Biotech) at 65 °C for at least 1 h in a buffer containing 1× SSC. 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-32P]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 2325 to 2880 and from positions 1678 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-32P]CTP. Full-length transcripts were isolated by preparative polyacrylamide gel electrophoresis and used for hybridization to DNA oligonucleotides as described above. In vitro transcription reactions contained 10 μl of buffer C (20 mM Tris/HCl, pH 7.3 (room temperature), 1.5 mM MgCl2, 10 mM KCl), incubated for 10 min on ice, and spun down at 2000 rpm in a Heraeus Varifuge 3.0R. Cells 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 and resuspended in 1.5 ml of buffer C (20 mM Tris/HCl, pH 7.3 (room temperature), 50 mM glycerol, 420 mM NaCl, 1.5 mM MgCl2, 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), 200 mM 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 MgCl2, 25 mM Hepes, pH 8.2, 5 mM dithiothreitol, 200 ng/ml bovine serum albumin (Roche Molecular Biochemicals), 1 mM methylbenzethionyl fluoride, 100 μM ATP, GTP, and UTP, respectively, 5 μM CTP, and 50 μCi of [α-32P]CTP (Amersham Pharmacia Biotech, 3000 Ci/mmole) 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 pH 8.0 and step-eluted with sodium chloride) and adsorbed to a mixture of 15 μl of glycogen and 5 μl of KCl in buffer C. The P11 system consisted of 50 μl 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 BB buffer (3 mM MgCl2, 10 mM NaCl, 10 mM Tris-HCl pH 7.4, and 0.1% Igepal CA-300 (Sigma)).
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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 EcoRI recognition sites (E2Fmt, ME1a1mt); the TATA box at 24 bp upstream of P2 was deleted (ΔP2TATA) (Fig. 1A 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 (ΔP1/P2, Fig. 1A).

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. 1A). 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. 2A). 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. 2A, 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. 2A, 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 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 (No Ab) and a reaction that lacked chromatin (Mock). Each of the antibodies was shown by Western blot analysis to detect its cognate DNA isoforms with blocked protein A-positive Staph A cells. Following extensive washing, bound 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 μl of immunoprecipitated chromatin, 1× Taq reaction buffer (Promega), 1.5 mM MgCl₂, 50 ng of each primer, 1.7 units of Taq polymerase (Promega), 200 μM each dNTP (Roche Molecular Biochemicals), and 1 mM 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 3 min, and 27 cycles of 95 °C for 1 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 epitope 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'-GGCCCTCTAGAGGCTTGGAA-3; Myc-2857, 5'-TCCAGCGTC-TAAGGAGCCTGCAAG-3; Myc-E2Fmt, 5'-GGCTCTCATAGAGGCTTGAA-3; Myc-ME1a1mt, 5'-GCTTGCAGGAGGAGGATTC-3. 

RESULTS
the P1 promoter in E2Fmt cells (lane 4) but not in ME1a1mt cells (lane 6). In ∆P2TATA 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, ∆P2TATA, and ∆P1/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. 3A). 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. 3A, lane 1) but strongly reduced in ME1a1mt and ∆P2TATA cells (lanes 3 and 4). In contrast, the density of pol II at the P2 promoter in E2Fmt cells was in-
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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 α[32P]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-32P]CTP. D, labeled RNAs were hybridized to oligonucleotides, and intensities of signals were evaluated as described above.

increased compared with wt cells (lane 2). Although the P1 wt promoter showed almost no pausing (Fig. 3A, 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. 3B). 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 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. 3C, 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. 3C, 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. 4L). A digest of 20 min produced mostly mono-, di-, and trinucleosomal fragments (Fig. 4L, 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. 4A, B, D, and E, lane 5). This pattern was restricted to five nucleosomes if DNA was cut with XbaI (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. 4C,
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 HindIII 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 AccI 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. 4H, lane 2). A significant change was also observed for the chromatin of ΔP1/P2 cells. These cells clearly established the nucleosome 3 (Fig. 4K, lane 2), which was only marginally detectable in chromatin of wt cells. If DNA was cut with the restriction enzyme PstI (Fig. 4O), 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. 4O.

Occupyance 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 promoter activity in vitro, 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
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-mycwt 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) and 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 knock-out 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. 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 is likely identical to 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 with 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).

**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.

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