The nucleosomal structure of active and inactive c-myc genes has been analyzed in detail in undifferentiated and differentiated cells of the promyelocytic leukemia cell line HL60. The c-myc P2 promoter was never found in nucleosomal configuration, no matter whether c-myc was expressed or not. Differences in the nucleosomal structure, however, were found in the promoter upstream region proximal to a previously described DNase I-hypersensitive site I, at the P0 promoter, and at the P1 promoter and upstream thereof. In these regions nucleosomes were detected in differentiated but not undifferentiated HL60 cells. Similar patterns of nucleosomes as found for active and inactive c-myc genes in HL60 cells were found for active and inactive episomal c-myc genes in stably transfected B cell lines. In these cell lines three activation stages could be described for episomal c-myc constructs: (i) uninducible, (ii) inducible, and (iii) induced. Significant differences in the nucleosomal structure of c-myc were observed for the uninducible and inducible stages, but not for the inducible and induced stages.

Activation of the proto-oncogene c-myc is consistently observed in a variety of tumors. Expression of c-myc has been shown to be regulated at the level of initiation, RNA elongation, RNA stability, and protein turnover. The gene is transcribed from three adjacent promoters P0, P1, and P2 and a fourth promoter, P3, in the first intron. RNA initiated at the promoter P2 usually contributes to 80–90% of total c-myc steady-state RNA in normal cells (1–4).

A variety of positive and negative elements has been identified within the regulatory region of the c-myc promoters with potential binding sites for the transcription factors NF1/CTF, SP1, E2F, Ap1, OCT, YY1, Maz, FBP, LR1, hu-CUT, CTCF, and others (5–22), for reviews, see Refs. 1 and 2. Whether all of these factors bind and contribute to c-myc regulation in vivo is still unclear. In addition, non-B DNA structures in the c-myc upstream region have been detected including triple helices, Z-DNA, and single stranded regions (23–26). Factors binding to non-B DNA structures may contribute to c-myc regulation in vivo as well. Expression of c-myc is also controlled at the level of RNA elongation (27–29). After initiation at the P2 promoter, RNA polymerase II (pol II) transcribes a short piece of RNA and then pauses ~10 to 40 bp downstream to the promoter (30, 31). Activation of these polymerases is suggested to occur by the action of transcriptional activators (32, 33) and to involve phosphorylation of the carboxyl-terminal domain of the large subunit of pol II (34).

Attempts to reconstitute c-myc regulation in transfected cell lines and transgenic mice carrying large fragments of the c-myc gene locus have been unsuccessful. The transfected or transgenic c-myc genes consistently turned out to be repressed and not inducible by stimuli that strongly induced the endogenous c-myc. This was surprising since most of the constructs displayed a bona fide chromatin structure when compared to the endogenous c-myc. The unresponsiveness of the exogeneous c-myc has been ascribed to a yet unidentified locus control/enhancer element of the c-myc gene locus which was missing on the constructs examined (35). Heterologous enhancers, e.g. the enhancer elements of the immunoglobulin (Ig) heavy and κ light chain gene loci, activated c-myc on DNA constructs in stably transfected cell lines and in transgenic mice quite efficiently (36–39). The κ intron- and κ 3′-enhancer have been shown to cooperate in c-myc activation. While the 3′-enhancer did not significantly activate c-myc, it strongly enhanced c-myc expression in combination with the intron enhancer (37, 39).

Defined nucleosomal structures of promoter regions constitute an essential regulatory mechanism of eukaryotic gene expression in vivo (40–42). One of the first steps in the activation process of a gene from a repressed state is the rearrangement or disruption of the chromatin structure, which otherwise prevents binding of the basal transcription machinery to the promoter. Access of transcriptional activators and the transcriptional machinery to chromatin-packed DNA is suggested to be enhanced by nucleosome-remodeling complexes such as SWI-SNF and the nucleosome-remodeling factor, NURF (43–46). A common pattern of DNase I-hypersensitive sites has been identified in cell lines expressing c-myc and changes in the pattern could be correlated with alterations in gene activity (27, 28, 47–50). In this report we show that changes in the sensitivity to DNase I in c-myc chromatin are accompanied by a remodeling of the nucleosomal structure in the c-myc promoter region.

MATERIALS AND METHODS

Cell Lines and Cell Culture—HL60 is a promyelocytic cell line carrying an amplified c-myc gene (51). Cell line RF266C3 was obtained by stable transfection of the Burkitt lymphoma cell line Raji with the construct RP261-4 (52) containing the 8.1-kilobase HindII-EcoRI c-myc gene locus (53) and the immunoglobulin κ-gene (Ig-k) 3′-enhancer (54) on an episomal, Epstein-Barr virus-derived vector pH2OPL (55, 56). Cell line MA76 was obtained by stable transfection of an Epstein-Barr virus immortalized lymphoblastoid B cell line with a c-myc construct lacking the Ig-k 3′-enhancer. Cell line CJ40 was obtained by stable transfection of Raji cells with a c-myc construct containing the 23-

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kilobase EcoRI-XhoI 3' -fragment of the c-myc gene instead of the Ig-k 3' enhancer (57). HL60 cells were grown to a density of 8 × 10^6 cells ml^{-1} in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin, streptomycin, and l-glutamine. For differentiation, HL60 cells were grown in the presence of 1.5% MeSO for 5 days.

Micrococcal Nuclease Treatment—Nuclei were isolated essentially according to a nuclear run-on protocol (28). Samples containing 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 time at room temperature with 3 units micrococcal nuclease (MNase) (Sigma, Deisenhofen, Germany). The reaction was stopped by the addition of 10 μl of 0.5 M EDTA and DNA was purified. DNA samples were chosen for further analysis, where 5–10 and 20–30% of nucleosomal spacers had been cut by MNase.

DNA Purification, Electrophoresis, and Southern Blotting—DNA was purified and examined by standard methods (58). DNA was digested with restriction enzymes under the conditions recommended by the manufacturer (New England Biolabs). DNA fragments were separated in a 2% agarose gel. DNA fragments were multiprime labeled with [α-32P]dCTP and used as hybridization probes. DNA probes were prehybridized in Church buffer (7% SDS, 0.5 M sodium phosphate buffer, 1 mM EDTA, pH 7.1) (59) for 30 min at 65°C. Hybridization was carried out for 24 h in fresh Church buffer after addition of the DNA probe. DNA probes were multiprime labeled with [α-32P]dCTP. After hybridization, the filter was washed several times at 50°C in a solution of 2× SSC, 1% SDS. Filters were exposed to Kodak X-Omat AR film at −80°C with intensifying screens.

DNA Probes—DNA probes were generated by polymerase chain reaction. The following primer pairs were used. Primer positions are given according to the sequence published by Gazin et al. (55). Probe A: 111/136–292/315, probe B: 830/853–1153/1177, probe C: 1153/1177–1343/1367, probe D: 1865/1890–2041/2065, probe E: 2981/2905–3061/3085, and probe F: 3235/3260–3410/3435. Polymerase chain reaction fragments were multiprime labeled with [α-32P]dCTP and used as hybridization probes.

RESULTS

Chromosomal and Episomal c-myc Genes Display Nucleosomal Structure—HL60 cells carry 20–30 copies of an amplified c-myc gene. The cells down-regulate c-myc transcription and undergo terminal differentiation toward granulocytes in the presence of 1.5% dimethyl sulfoxide (MeSO) in the culture medium. Changes in chromatin structure accompanying down-regulation of c-myc in HL60 cells have been studied in detail with DNase I before (27, 28, 48, 50). The early phase of c-myc repression within the first hours occurs without significant changes in the chromatin structure, as revealed by the continuous presence of DNase I hypersensitive sites I, II_2, III_1, and III_2. In the time course of differentiation c-myc chromatin becomes more resistant and after 5 days, sites II_2, III_1, and III_2 disappear almost completely, while sites I and III_3 remain unaffected (50). DNase I-hypersensitive sites have also been studied for stably transfected, episomal c-myc constructs in B cell lines. The episomal c-myc in MA76 cells (Fig. 1A) establishes a chromatin with hypersensitive sites similar to differentiated HL60 cells with undetectable sites II_2, III_1, and III_2. In RF266C3 cells (Fig. 1B), the episomal c-myc establishes a chromatin similar to proliferating HL60 cells with sites I, II_2, and III_3 (52, 57; data not shown). The pattern of hypersensitive sites in CJ40 has not been studied before. The episomal c-myc constructs in MA76, RF266C3, and CJ40 cells are consistently found to be repressed (52, 60, data not shown). Sodium butyrate strongly induces the episomal c-myc in RF266C3 cells (52) but not in MA76 and CJ40 cells (data not shown).

The sensitivity of chromatin to MNase can roughly be divided into three classes: insensitive, sensitive, and hypersensitive. While DNA packed in nucleosomes usually is insensitive to MNase digestion, the spacer sequence of approximately 20 bp length between nucleosomes is sensitive and gives rise to a nucleosomal ladder after partial MNase digestion (Fig. 3G). MNase-hypersensitive chromatin contains either extended regions of nucleosome-free DNA or particular DNA structures that promote MNase attack. Nucleosomal ladders are interrupted at MNase-hypersensitive sites. To study the nucleosomal structure of c-myc in the various cell lines, nuclei were isolated and incubated with MNase for increasing periods of times. In the presented experiments three time points of these kinetics are shown: (i) untreated chromatin, chromatin treated with MNase until (ii) 5–10% or (iii) 20–30% of nucleosomal spacers had been cut. DNA was purified and fragments were separated on a 2% agarose gel (Fig. 3G, lanes 4–6), transferred to membrane filters and hybridized with various radioactively labeled small DNA probes homologous to sequences upstream and downstream of the P1/P2 promoter region (Fig. 2). Probes A–F detected nucleosomal ladders in all cell lines indicating the presence of nucleosomes upstream and downstream of the P1/P2 promoters, irrespective of the chromosomal or episomal status of c-myc (Figs. 3–7).

Positioned Nucleosomes Downstream of the Dual c-myc Promoters P1/P2—We next asked whether nucleosomes in the c-myc promoter region have fixed positions. DNA samples were cut with various restriction endonucleases as indicated in Fig. 2. Hybridization experiments were performed with DNA probes located immediately upstream or downstream of a restriction endonuclease cut site, allowing for a precise mapping of nucleosome positions. For convenience, the positions of individual nucleosomes determined in this study have been marked by consecutive numbers starting at the HindIII site (Fig. 2). A ladder of five nucleosomes (nucleosomes 14–18) was detected when DNA of HL60 cells was digested with XhoI and hybridized with probe F (Fig. 3A, lane 2). Accordingly, a ladder...
of three nucleosomes was detected after XmnI digestion and hybridization with probe E (Fig. 3F, lane 2). The observed interruption of the nucleosomal ladder proximal to the P2 promoter is in line with the detection of paused transcription complexes at this position in HL60 cells (30, 31). Without XbaI digestion probe F detected at least seven nucleosomes indicating that the first exon and a large portion of the first intron of c-myc have a regular nucleosomal structure (Fig. 3A, lane 5). The pattern of nucleosomes downstream of the P2 promoter did not change in differentiated HL60 cells 5 days after addition of Me2SO (Fig. 3B).

The episomal c-myc genes in cell lines MA76, RF266C3, and CJ40 revealed the same nucleosomal pattern downstream of the P2 promoter as detected in HL60 cells (Fig. 3, C–E). Again, the ladder was interrupted proximal to the P2 promoter consistent with the pausing of pol II complexes on episomal constructs in RF266C3 cells (34, 52). Promoter proximal pausing of pol II in MA76 and CJ40 cells is currently under investigation. Activation of paused transcription complexes by sodium butyrate in RF266C3 cells (52) did not affect the nucleosomal pattern downstream of the P2 promoter (data not shown).

**Nucleosomal Structure at DNase I-hypersensitive Site I**—The upstream region of an active/induced c-myc gene is characterized by the presence of DNase I-hypersensitive sites I, II, and III. DNAs were purified and subjected to Southern analysis either undigested (G, lanes 4–6) or digested with the restriction endonucleases XhoI (G, lanes 1–3) or XmnI. DNAs were hybridized with probe F (A–E) and probe E (F). Nucleosomes detected in lane 2 of each panel are shown schematically at the right-hand side of the autoradiograms and numbered consecutively as shown in Fig. 2. Cuts of nucleosomal spacer regions are indicated by solid arrowheads. M designates a lane with marker fragments.
markedly. Digestion of the DNA with Kpn1 generated a ladder of four nucleosomes extending from nucleosome 7 to nucleosome 4 (Fig. 5A, lane 2), indicating the absence of nucleosome 3 from chromatin of proliferating HL60 cells. In addition, the spacer between nucleosomes 4 and 5 appeared almost entirely resistant to MNase-digestion in untreated HL60 cells (Fig. 5A, lane 2, open triangle). These peculiar differences in chromatin of untreated and MeSO-treated HL60 cells were also observed for episomal c-myc in MA76 and RF266C3 cells. The nucleosomal pattern detected by probe B in MA76 cells was almost identical to the pattern in MeSO-treated HL60 cells (Fig. 5C), whereas the nucleosomal pattern in RF266C3 cells was identical to the pattern in untreated HL60 cells (Fig. 5D).

**Nucleosomal Structure at DNase I-hypersensitive Site II**—The pattern of nucleosomes upstream of HS II was studied with probe C. Probe C detected a ladder of three nucleosomes in uncut DNA of HL60 cells, which consists of nucleosomes 8, 7, and 6 (Fig. 6A, lane 5). The extension of this ladder to nucleosomes 5 and 4 (open triangle) described above. Restriction of HL60 DNA with ClaI shortened the ladder to nucleosomes 8 and 7 and a small portion of nucleosome 6 (Fig. 6A, lane 2). This reveals a MNase-hypersensitive site downstream of nucleosome 8 near to DNase I-hypersensitive site II. The hypersensitivity of this site may be explained by the absence of nucleosome 9 from chromatin of proliferating HL60 cells. This appears likely, since nucleosome 9 would cover the potential pol II entry site at promoter P0, and because P0 RNA is readily detectable in growing HL60. The pattern of nucleosomes detected by probe C in MeSO-treated HL60 cells differed from the pattern detected in untreated cells. After ClaI restriction, probe C detected a nucleosomal ladder extending from nucleosome 7 to nucleosome 13 (Fig. 6B, lane 2). In contrast to uninduced HL60 cells, nucleosome 9 was clearly detectable in the chromatin of differentiated HL60 cells. Evidence for the presence of nucleosome 9 was also found for the episomal c-myc in MA76 cells (Fig. 6C, lane 2) but not for the episomal c-myc in RF266C3 cells (Fig. 6D, lane 2).

**Nucleosomal Structure Upstream of DNase I-hypersensitive Sites III**—Hybridization with probe D confirmed the reduced sensitivity of the P0 promoter region to MNase in differentiated HL60 cells. Probe D detected a large nucleosomal ladder in uncut DNA (Fig. 7B, lane 2). This ladder was shortened to 4 nucleosomes after restriction with AccI (lane 2), indicating an interruption in the ladder downstream of nucleosome 13. Nucleosome 12 was only faintly visible in the nucleosomal ladder possibly due to a reduced sensitivity of the spacer region between nucleosome 12 and 13 to MNase (Fig. 7B, lane 2, open triangle). While nucleosome 13 was clearly visible in chromatin of differentiated HL60 cells, this nucleosome was detectable only in minor amounts in chromatin of proliferating HL60 cells (Fig. 7A, lane 2, submolar amounts of nucleosomes are indicated by dashed lines). Nucleosomes 12 and 13 were also barely detectable in chromatin of episomal c-myc in RF266C3 cells (Fig. 7D, lane 2) but clearly visible in chromatin of episomal c-myc in MA76 cells (Fig. 7C, lane 2). The absence of nucleosomes 12 and 13 from chromatin of RF266C3 cells was confirmed after restriction of DNA with PstI and hybridization with probe D. Probe D detected nucleosome 11 proximal to the PstI site but did not detect nucleosomes 12 and 13 further downstream (Fig. 7E, lane 2). Thus, the presence and absence of hypersensitive sites III1 and III2 in c-myc chromatin of HL60 cells reported previously correlates with the presence and absence of nucleosomes 12 and 13. Importantly, the cap site of the P2 promoter and adjacent sequences were never found in nucleosomal configuration, irrespective of the chromosomal or episomal status of c-myc and irrespective of whether c-myc was expressed or not. A summary of the different nucleosomal structures found in HL60, MA76, RF266C3, and CJ40 cells is shown in Fig. 8.

**Reduced Sensitivity of Nucleosomal Spacer Regions to MNase**—In addition to the interruptions of the nucleosomal ladder in the c-myc promoter region described above, we were able to define a further characteristic feature of c-myc chromatin. Several nucleosomal spacer regions with reduced sensitivity to MNase were observed. One potential spacer region with reduced sensitivity was detected between nucleosomes 12 and 13 in HL60 and MA76 cells (Figs. 6, B and C, and 7, A-C, open triangles). Since this spacer never displayed a regular sensitivity to MNase, it remains questionable whether the insensitivity...
to MNase is in fact brought about by cellular factors binding to the potential spacer region. Alternatively, the P1 promoter upstream region may contain a chromatin free of nucleosomal structure.

The second spacer region resistant to MNase digestion was detected between nucleosomes 4 and 5. Interestingly, this spacer was only protected in proliferating HL60 cells and on episomal c-myc in RF266C3 cells (Fig. 5, A and D, lane 2, open triangle), but showed normal sensitivity toward MNase in Me2SO-treated HL60 cells and in MA76 cells (Fig. 5, B and C, lane 2). The reduced accessibility of the spacer to MNase correlates with the expression and inducibility of c-myc in HL60 and RF266C3 cells, respectively.

**DISCUSSION**

The nucleosomal structure of chromosomal and episomal c-myc promoter regions was delineated by indirect end label mapping of sites accessible to MNase. By this method nucleosomes were mapped in relation to the various transcriptional start sites and DNase I-hypersensitive sites in chromatin of a transcriptionally active c-myc clone. Starting with nucleosome 1 adjacent to the HindIII site (+1), 18 nucleosomes were mapped down to the XbaI site (+3508) in the first intron of c-myc. Additionally, a nucleosome-free region of -180 bp was detected at the P2 promoter. By estimating the sequence required for a single nucleosome and a spacer region of -180 bp, the calculated number of nucleosomes that maximally can bind the single nucleosome and a spacer region of 180 bp was estimated as 12.

The mapping of MNase-hypersensitive sites in this study now allows us to make more precise statements about the nature of DNase I-hypersensitive sites. The presence of HS I in chromatin of inactive c-myc alleles has previously been assumed as indicative for a negative regulatory role of this site in c-myc expression (47). Here we have shown that this site is also hypersensitive to MNase in the cell lines analyzed. In addition, this study has revealed differences downstream of site I in the chromatin of c-myc which remained uncovered by DNase I analyses. While a regular ladder of nucleosomes was detected downstream of site I in chromatin of MA76 and Me2SO-treated HL60 cells, this ladder was significantly altered in RF266C3, CJ40, and untreated HL60 cells. Two major alterations were clearly visible: (i) cutting of the spacer between nucleosomes 4 and 5 was blocked, and (ii) nucleosome 3 appeared to be absent. A far upstream stimulating element has been described in c-myc that co-localizes with the spacer region between nucleosomes 4 and 5 (24). This element displays single-stranded conformation and binding of the single-strand specific transcriptional activator FBP when c-myc is in an activated stage. However, the element is double stranded if c-myc is in an inactive stage (16, 26). Interestingly, c-myc and FBP RNA levels are co-regulated in HL60 cells and decrease with similar kinetics after induction of differentiation (20). Whether binding of FBP to the sense strand can inhibit MNase attack is not yet known. Changes in chromatin structure observed at the far upstream stimulating element site may be linked to the disruption of nucleosome 3 further upstream. In RF266C3, CJ40, and untreated HL60 cells the absence of nucleosome 3 and the resistance of the spacer region between nucleosomes 4 and 5 to MNase are observed, while in MA76 and Me2SO-treated HL60 cells neither of these two changes occur. Finally, the spacer region between nucleosomes 2 and 3 was found to be hypersensitive to MNase and DNase I in all cells. Thus, it is possible that this region may serve as a starting point for organization and remodeling of c-myc chromatin further downstream.
relevance of this site for c-myc regulation in vivo may have been underestimated in the past. Mutagenesis and reconstruction of the chromatin structure on episomal DNA constructs will help to study the role of this site for c-myc regulation.

The potential entry sites for RNA polymerase II at the promoters P0, P1, and P2 were not occupied by nucleosomes in RF266C3, C340, and untreated HL60 cells. In contrast, the P0 and P1 promoters, but not the P2 promoter, were occupied by nucleosomes in MA76 and MeSO-treated HL60 cells. This points to different modes of repression for the different c-myc promoters. In fact, repression of promoters P1 and P2 have been reported to occur at different levels in terminally differentiating mouse erythroleukemia cells. While the P1 promoter is repressed at the level of initiation, repression of the P2 promoter occurs by promoter proximal pausing of pol II (61). Repression of the P2 promoter in differentiating HL60 cells has also been reported to be mediated by promoter proximal pausing of pol II (30, 31). The mechanism of P1 repression in these cells is not yet clear. The binding sites for the majority of potential c-myc transcription factors lie upstream of P1 in a region corresponding to nucleosomes 12 and 13. It will be necessary to study whether these factors can bind to nucleosomal DNA and, if so, whether factor binding affects the nucleosomal structure.

Notably, DNA sequences encompassing DNaSe I-hypersensitive site II are evolutionary less conserved between mouse than sequences encompassing sites I and III1–3 (49).

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