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Nucleosomal Structures of c-*myc* Promoters with Transcriptionally Engaged RNA Polymerase II

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Organization of DNA into chromatin has been shown to contribute to a repressed state of gene transcription. Disruption of nucleosomal structure is observed in response to gene induction, suggesting a model in which RNA polymerase II (pol II) is recruited to the promoter upon reorganization of nucleosomes. Here we show that induction of c-*myc* transcription correlates with the disruption of two nucleosomes in the upstream promoter region. This nucleosomal disruption, however, is not necessary for the binding of pol II to the promoter. Transcriptionally engaged pol II complexes can be detected when the upstream chromatin is in a more closed configuration. Thus, upstream chromatin opening is suggested to affect activation of promoter-bound pol II rather than entry of polymerases into the promoter. Interestingly, pol II complexes are detectable in both sense and antisense transcriptional directions, but only complexes in the sense direction respond to activation signals resulting in processive transcription.

Chromatin but not naked DNA is the physiological template for the transcription process, and the chromatin structure itself has a major influence on this process. The regulatory regions of many genes are organized in phased nucleosomal arrays. The defined nucleosomal structure of promoter regions constitutes an essential regulatory mechanism of eukaryotic gene expression in vivo (16, 46). Accumulating evidence suggests a fine and balanced tuning between the chromosomal environment and the transcriptional process (1, 69, 79).

Biochemical and genetic analyses in yeast identified nucleosomes as general repressors of transcription (19, 21, 26). Gene activation is accompanied by perturbations or alterations of the nucleosomal structure. A first critical step for activating a gene locus seems to be liberating the promoter region from histone-mediated repression (6, 69). Disruption of nucleosomal structure in response to gene activation has been observed at the *Saccharomyces cerevisiae PHO5*, *GAL1* and *ADH2* promoters (5, 61, 74, 76). A similar activation mode has been reported for inducible proviral promoters of mouse mammary tumor virus (2, 3, 48, 56, 72) and of human immunodeficiency virus type 1 (47, 73, 75). In these examples, the transcription complex seems to be recruited to the promoter only after positioned nucleosomes have been disrupted by the concerted action of invading chromatin remodeling and *trans*-acting factors.

An increasing number of genes, including heat shock genes (58, 59), c-*fos* (49), and c-*myc* (25, 27, 42, 66, 67), have been shown to be regulated by promoter-proximal pausing of RNA polymerase II (pol II). Interestingly, pol II density over the pause site is unchanged upon gene induction, suggesting that elongation of polymerases from the pause site is tightly coupled with the loading of pol II onto the pause site. Promoter-proximal pausing has been studied extensively for the c-*myc* gene. In human cells, this gene is transcribed from the promoters P0, P1, P2, and P3, with preferential usage of P2. Pol II initiates transcription at the P2 promoter but pauses approximately 10 to 40 nucleotides downstream thereof (27, 66). Activation of these stalled transcription complexes is thought to be mediated through the action of transcriptional activators (80) and enhancers (28) by increasing the elongation competence of pol II. Recent findings suggest an involvement of signals which enhance phosphorylation of the carboxy-terminal domain of the large subunit of pol II (78). Factors binding to or being released from the carboxy-terminal domain not only may be involved in the regulation of pol II processivity but have recently been shown to be also necessary for successful processing and splicing of the nascent transcript (36). In murine plasmacytoma and Burkitt's lymphoma cells, c-*myc* is constantly juxtaposed to one of the immunoglobulin (Ig) gene loci. The translocated allele in these cells displays a functional lack of the block to RNA elongation (9, 14, 67). Combinations of various enhancer elements and locus control regions of the Ig heavy-chain and κ light-chain gene loci have been demonstrated to activate c-*myc* on DNA constructs with characteristics similar to that observed for the translocated allele (22, 33, 52).

Differences in chromatin structure have been described for inactive and active c-*myc* genes (12, 15, 63, 64). Depending on its transcriptional activity, the c-*myc* chromatin displays different degrees of susceptibility to DNase I cleavage. The patterns of DNase I-hypersensitive sites (HSs) are thought to correspond to putative regulatory elements within the promoter and 5′ flanking region where a variety of positive and negative *cis* elements have been identified (34, 65). However, investigations to determine the factors and *cis* elements that activate c-*myc* expression in vivo have been unsuccessful. Analyses of mice transgenic for various parts of the c-*myc* gene revealed that the *cis* elements described to drive correct c-*myc* transcription in vitro are not sufficient in vivo (29).

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Here we have studied promoter-proximal pausing of pol II on episomal c-*myc* constructs stably transfected in different B-cell lines. While all constructs were able to establish promoter-proximal pausing of pol II, the overall chromatin structure and inducibility of paused pol II on the episomal constructs were dependent on the cellular background.

MATERIALS AND METHODS

Cell lines and cell culture. Cell line RF266C3 was previously described in detail (67). It was obtained by stable transfection of Raji cells with the DNA construct RF261-4 (67) containing the 8.1-kb *Hin*dIII-*Eco*RI c-*myc* gene locus and in addition a 4.1-kb *Bam*HI fragment encompassing the human Ig-κ 3′ enhancer on the episomal, self-replicating vector pHEBOPL (51, 68). Raji is an Epstein-Barr virus EBV-positive Burkitt's lymphoma cell line with a t(8;14) translocation (50). Cell line MA76 was established by stable transfection of LCL721 cells with a c-*myc* construct which lacks the Ig κ 3′ enhancer (κ3′E). LCL721 is an EBV-immortalized lymphoblastoid cell line (24). Transfection of Raji cells with the enhancerless construct gave rise to cell line KH375. HL60 is a promyelocytic cell line carrying an amplified c-*myc* gene which can be induced to differentiate into granulocytes by dimethyl sulfoxide (DMSO) (10, 11). All cells were grown to a density of 10^6/ml in 10% fetal calf serum–RPMI 1640 medium (Life Technologies, Gibco BRL) supplemented with penicillin, streptomycin, and L-glutamine. Medium for the c-*myc* transfectants contained in addition 300 μg of hygromycin B per ml.

Preparation of total cellular RNA. Approximately 10^8 cells were washed with ice-cold phosphate-buffered saline and spun down at 1,200 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 previously (60).

Preparation of nuclei. Nuclei were isolated as described previously (13). Briefly, cells were spun down at 1,200 rpm for 10 min at 4°C and washed twice with ice-cold phosphate-buffered saline, and the pellets of 10^8 cells were resuspended in 10 mM Tris HCl (pH 7.4)–10 mM NaCl–3 mM $MgCl_2$–0.5% (vol/vol) Nonidet P-40. After incubation on ice for 5 min, the lysate was spun down at 1,500 rpm for 15 min at 4°C. The pelleted nuclei were resuspended in storage buffer (50 mM Tris HCl [pH 8.3], 40% [vol/vol] 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 an M13 clone. Hybridization of labeled DNA fragments to RNA was carried out as described by Berk and Sharp (8), with slight modifications. Hybridization mixtures of 30 μl containing 10^5 cpm of the probe (specific activity, 10^8 cpm/μg), 30 μg of RNA, 90% (vol/vol) formamide, 400 mM NaCl, 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (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 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% (vol/vol) glycerol, and 400 U of nuclease S1 (Boehringer Mannheim). The samples were incubated at 25°C for 1 h, extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol), and precipitated with ethanol. Protected DNA fragments were separated on 5% (wt/vol) polyacrylamide gels.

Mapping of HSs. Preparation of DNase I-treated nuclei, gel electrophoresis, and hybridization procedures were performed essentially as described previously (23, 35). The hybridization probe is a multiprime-labeled PCR fragment, located within the first intron of c-*myc* (positions 3264 to 3810) (18).

Mapping of nucleosomes by MNase. Nuclei were isolated essentially as described above. Nuclei (2×10^7) 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 U of micrococcal nuclease (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 of 0.5 M EDTA, and DNA was purified as described previously (60). For further analysis, we chose DNA samples in which approximately 5 to 10% and 30 to 40% of nucleosomal spacers had been cut by MNase. In addition, nuclease-digested DNA was cut with restriction endonuclease *Xba*I, *Acc*I, or *Pst*I in conditions recommended by the manufacturer (New England Biolabs). DNA fragments were separated in a 2% (wt/vol) agarose gel, denatured by alkali treatment, transferred to a nylon membrane (Hybond N+; Amersham), and hybridized with multiprime-labeled PCR probes. Hybridization probes L (positions 1865 to 2065) (18) and R (positions 2881 to 3085) were generated by PCR using specific c-*myc* primers and multiprime labeled with [α-^{32}P]dCTP. Hybridization was carried out for 24 h in Church buffer (7% [wt/vol] sodium dodecyl sulfate [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 procedure for washing of membranes, including the digestion of single-stranded RNA with RNase A, have been described in detail elsewhere (13, 66, 67). Briefly, 100 μl, corresponding to 2×10^7 isolated nuclei in storage buffer (50 mM Tris HCl [pH 8.3], 40% [vol/vol] glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA), was 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 [each] ATP, GTP, and UTP; 100 μCi of [α-^{32}P]CTP [800 Ci/mmol; Amersham]) 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) at 65°C for at least 48 h in 5 ml of Church buffer. After washing and drying, the membranes were exposed to Kodak X-Omat AR film at −80°C with intensifying screens. The intensities of hybridization signals were determined with a BAS 1000 phosphorimager system (Fuji) and calculated relative 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 c-*myc* sense and antisense strands were synthesized on the basis of the sequence described in reference 18. The positions for antisense oligonucleotides A to M are as follows: A, 2278 to 2327; B, 2328 to 2377; C, 2378 to 2427; D, 2440 to 2489; E, 2490 to 2539; F, 2540 to 2589; G, 2590 to 2639; H, 2640 to 2689; I, 2690 to 2739; K, 2740 to 2789; L, 2790 to 2839; and M, 2840 to 2889. The positions for sense oligonucleotides A′ to O′ are as follows: A′, 2638 to 2589; B′, 2588 to 2539; C′, 2538 to 2489; D′, 2477 to 2428; E′, 2427 to 2378; F′, 2377 to 2328; G′, 2327 to 2278; H′, 2277 to 2228; I′, 2227 to 2178; K′, 2177 to 2128; L′, 2127 to 2087; M′, 2077 to 2028; N′, 2027 to 1978; and O′, 1977 to 1928.

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 (Boehringer Mannheim). 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 vivo footprinting with $KMnO_4$. Nuclei at a concentration of 2×10^8/ml were resuspended in nuclear run-on buffer in the presence or absence of nucleoside triphosphates (NTPs), and a run-on reaction was performed. Subsequently, nuclei were exposed to freshly prepared 10 mM potassium permanganate ($KMnO_4$) for 2 min at 37°C. Reactions were stopped by the addition of 1 ml of lysis buffer (300 mM LiCl, 10 mM Tris HCl [pH 8.0], 1 mM EDTA, 2% [wt/vol] SDS, 200 μg of proteinase K per ml) containing 2% (vol/vol) β-mercaptoethanol, and mixtures were incubated at 55°C for 2 h. In vitro modification of DNA, purification, and cleavage at modified base residues were done essentially as described previously (17). All DNAs were subjected to linker/ligation-mediated PCR (39). Primers used to footprint the c-*myc* P2 promoter region and hybridization temperatures were as described elsewhere (27).

RESULTS

Activation stages of episomal c-*myc* genes. Expression of the c-*myc* gene was studied in three human B-cell lines stably transfected with DNA constructs which contain an 8-kb fragment of the human c-*myc* gene together with the EBV origin of replication (oriP) to allow episomal propagation in EBV-positive cells (Fig. 1A). To prevent 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 (52). Cell line RF266C3 (67) is derived from the Burkitt's lymphoma line Raji and contains episomal constructs carrying the c-*myc* cloned adjacent to a 4-kb fragment encompassing the κ3′E. In addition, Raji cells were transfected with a construct lacking the κ3′E, giving rise to cell line KH375. The same construct was also transfected into the lymphoblastoid cell line LCL721, giving rise to cell line MA76. All three cell lines carried approximately 30 to 50 copies of the respective constructs (reference 67 and data not shown).

When expression of the episomal c-*myc* genes was studied by nuclease S1 analysis, constructs of all three cell lines were found to be expressed at very low levels (Fig. 1B, lanes 1, 3, and 5). It has been demonstrated previously that the episomal c-*myc* in RF266C3 cells is inducible by sodium butyrate (SoB) (67, 78) (Fig. 1B, lane 2). The c-*myc* construct lacking the κ3′E turned out to be uninducible in MA76 cells (Fig. 1B, lane 4). However, this construct was strongly induced in SoB-treated

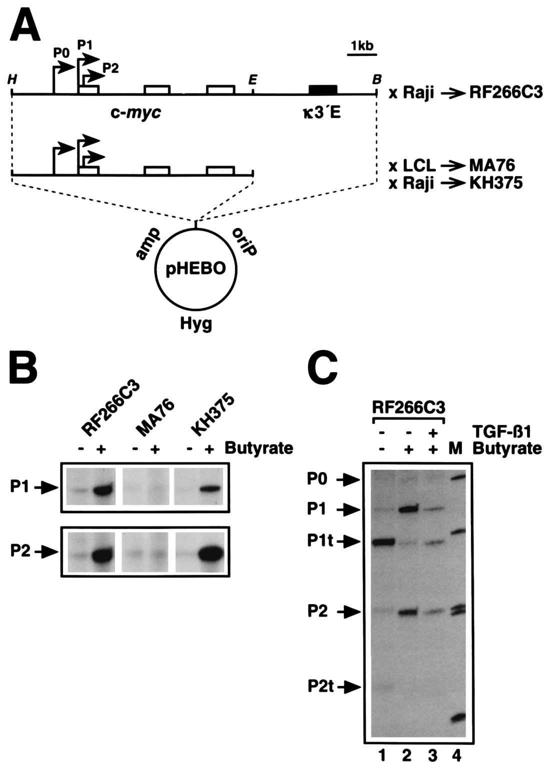

FIG. 1. Expression of episomal c-*myc* genes in stably transfected B-cell lines. (A) Episomal, pHEBO-derived c-*myc* constructs were used for stable transfections. Open boxes, c-*myc* exons 1 to 3; filled box, κ3′E; *H*, *Hin*dIII; *E*, *Eco*RI; *B*, *Bam*HI. All constructs carry the origin for episomal replication of EBV (oriP), the ampicillin resistance gene (amp), and the gene for hygromycin resistance (Hyg). LCL, lymphoblastoid cell line. (B) Analysis of RNAs expressed from stably transfected c-*myc* constructs in cell lines RF266C3, MA76, and KH375. All three cell lines contain approximately 30 to 50 episomal copies of the corresponding construct. Cells were cultivated in the absence (−) or presence (+) of 3 mM SoB for 24 h. Total RNA was isolated, and c-*myc* RNA was studied by nuclease S1 analysis. The probe discriminated RNAs derived from the endogenous c-*myc* alleles (not shown) and RNAs derived from the transfected constructs (P1 and P2). (C) Analysis of various c-*myc* transcripts in RF266C3 cells. RNA of untreated cells (lane 1), of cells cultivated in the presence of 3 mM SoB (lane 2), and of cells cultivated in the presence of TGF-β1 (10 ng/ml; Life Technologies) and SoB for 24 h (lane 3) was isolated and subjected to nuclease S1 analysis. P1t and P2t, transcripts derived from the endogenous translocated c-*myc* allele; P0, P1, and P2, transcripts derived from the episomal c-*myc*. Lane 4, [α-^{32}P]dCTP-labeled molecular weight standard (M).

KH375 cells (Fig. 1B, lane 6). Thus, the constructs with and without the κ3′E were inducible in the Raji background to similar extents. Therefore, the general inducibility of the different constructs depends on the cellular background rather than on the presence or absence of the κ3′E on the construct.

The mechanism of c-*myc* induction by SoB is not well understood and may involve modification of chromatin. SoB is known to interfere with histone deacetylation (57) and could thereby activate the episomal c-*myc* in RF266C3 and KH375 cells in an unspecific manner. Transcriptional activation of a normal c-*myc* in B cells is under the negative control of transforming growth factor β1 (TGF-β1) (32). We therefore tested whether induction of the episomal c-*myc* by SoB is inhibited by TGF-β1. Induction of transcripts derived from the episomal c-*myc* gene in RF266C3 cells (Fig. 1C, lane 2, P1 and P2) was largely inhibited in the presence of TGF-β1 (Fig. 1C, lane 3), which indicates that the activation mechanism by SoB is still under the negative control of TGF-β1 signalling.

Chromatin structure of episomal c-*myc* genes. The different inducibilities of c-*myc* constructs may rely on different responses to SoB in the two cellular backgrounds. Alternatively, identical c-*myc* constructs may establish different chromatin structures that allow or inhibit c-*myc* induction. To study the chromatin structures of episomal c-*myc* genes in the three cell lines, nuclei were prepared and digested with DNase I. DNA was isolated and cleaved with the restriction endonuclease *Afl*II, and the locations of HSs were mapped by Southern blot hybridization. For the episomal c-*myc* in MA76 cells, only HS I was detectable, indicative of the chromatin structure of a repressed c-*myc* gene (Fig. 2A). Even under conditions where the genomic fragment was almost completely digested, only HS I could be visualized by DNase I treatment. In contrast, the transfected gene in KH375 and RF266C3 cells adopted a chromatin structure characteristic of an expressed chromosomal c-*myc* gene (12, 63, 64) with HSs I to III_3 (Fig. 2A). This result illustrates differences of the episomal chromatin upstream of the c-*myc* promoter in MA76 cells versus KH375 and RF266C3 cells.

The chromatin structure within the c-*myc* promoter region was also studied after MNase digestion. Nuclei of the various cell lines were treated with MNase for increasing periods of time; DNA was purified and separated by gel electrophoresis. The resulting DNA fragments displayed the characteristic nucleosomal ladder (Fig. 3N). Digestion for 20 min produced mostly mono- and dinucleosomal fragments (Fig. 3N, lane 6) shorter than fragments obtained after a 3-min digestion (Fig. 3N, lane 5). The same pattern emerged when DNA in addition to MNase was cut with a restriction endonuclease (Fig. 3N, lanes 2 and 3). DNA fragments were hybridized with the ra-

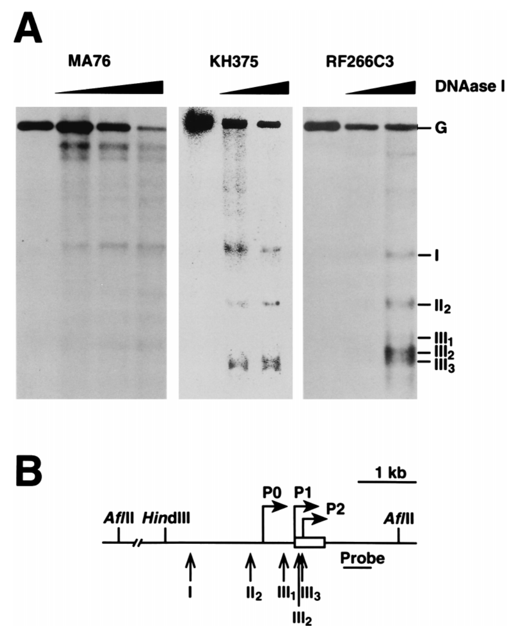

FIG. 2. Patterns of HSs of c-*myc* chromatin on episomal constructs in MA76, KH375, and RF266C3 cells. (A) Nuclei were prepared from all three cell lines and incubated with increasing amounts of DNase I (indicated by wedges). After DNA purification, 10 μg of each sample was digested with *Afl*II, separated by gel electrophoresis, blotted, and hybridized to a probe specific for c-*myc* intron 1 sequences. Besides the *Afl*II-*Afl*II genomic fragment (G), partial cleavage generates additional subbands corresponding to HSs I to III_3. (B) Map illustrating the locations of HSs (indicated by arrows). Exon 1 of c-*myc* is shown as a box. The relative locations of the promoters P0, P1, and P2 and of restriction endonuclease cleavage sites are indicated.

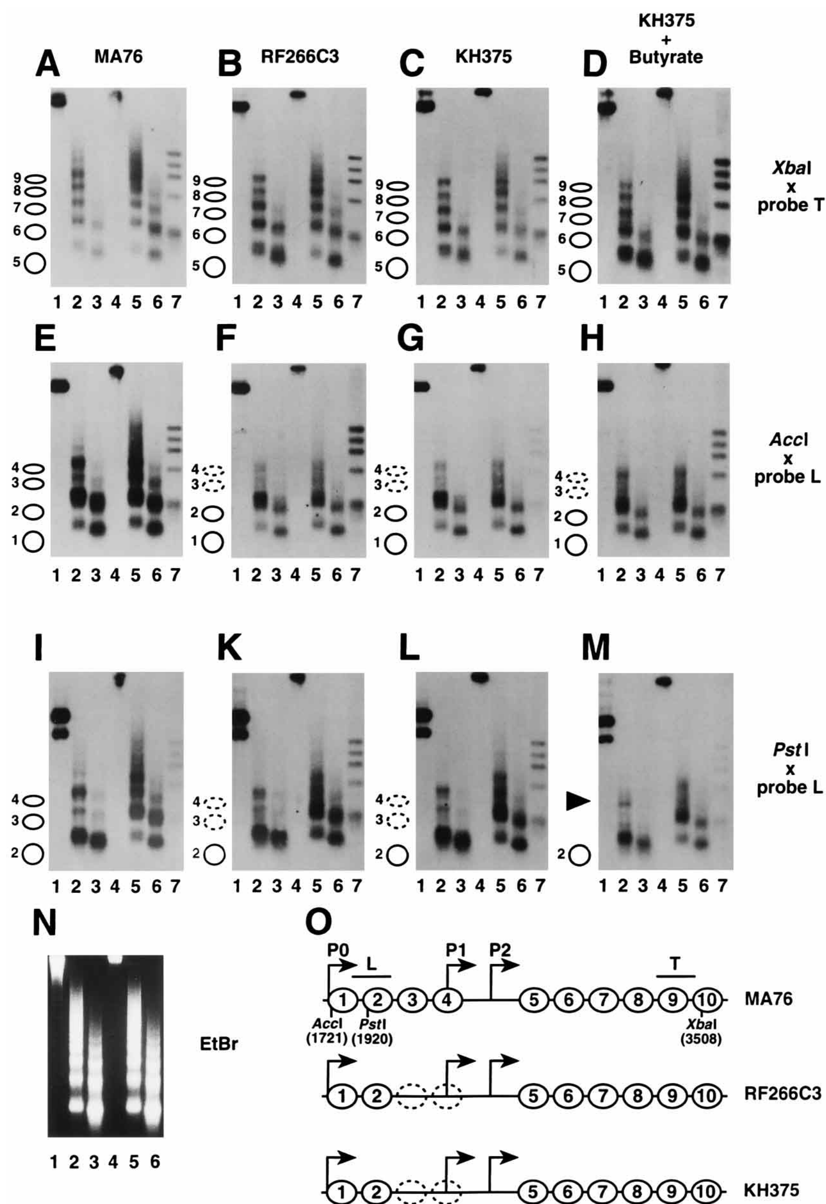

FIG. 3. Nucleosomal patterns upstream and downstream of the c-*myc* P2 promoter on episomal constructs in cell lines MA76, RF266C3, and KH375. (A to M) Southern blot analysis of MNase-digested DNA of MA76, RF266C3, and KH375 cells. (N) Ethidium bromide (EtBr) staining of nucleosomal DNA. Nuclei were isolated and incubated with MNase for different periods of time. Subsequently, DNA was purified and subjected to Southern analysis either uncut (lanes 4 to 6) or cut with restriction endonuclease *Acc*I, *Pst*I, or *Xba*I as indicated to the right of each row (lanes 1 to 3). The PCR fragments L and T were used as hybridization probes for nucleosomal patterns upstream (probe 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; lane 7, labeled φX174 DNA-*Hae*III digest (New England Biolabs), used as a molecular weight standard. Nucleosomes detected after restriction enzyme digestion are schematically shown on the left of each panel. (O) Scheme depicting the relative locations of nucleosomes and probes L and T within the c-*myc* promoter region. The positions of restriction endonuclease recognition sites refer to the previously published c-*myc* sequence (18).

dioactively labeled probe T, homologous to sequences downstream of the P2 promoter region. Using this probe, we detected more than five nucleosomes in MA76, RF266C3, and KH375 cells (Fig. 3A to C, lanes 5). This pattern was restricted to five nucleosomes when DNA was cut with *Xba*I (Fig. 3A to C, lanes 2), indicating that the region 70 to 100 bp downstream of the P2 cap site was hypersensitive to MNase in all three cell lines. Partial digestion of naked cellular DNA with MNase did not produce a nucleosomal ladder after hybridization with probe T (data not shown). Differences in c-*myc* chromatin between MA76 cells versus RF266C3 and KH375 cells were observed after hybridization with probe L upstream of P2. This probe detected four nucleosomes downstream of the *Acc*I site (nucleosomes 1 to 4) in chromatin of MA76 cells (Fig. 3E, lane 2). When DNA of MA76 cells was cut with *Pst*I, the positions of nucleosomes 2 to 4 downstream of the *Pst*I site were confirmed (Fig. 3I, lane 2). An HS beyond nucleosome 4 led to an interruption of the ladder. In RF266C3 and KH375 cells, nucleosomes 1 and 2 could clearly be detected downstream of the *Acc*I site by hybridization with probe L (Fig. 3F and G, lanes

2). However, hybridization signals corresponding to nucleosomes 3 and 4 were only faintly visible, indicating that most of the episomal constructs in RF266C3 and KH375 cells have no regular nucleosomal structure upstream of the P1 promoter. The nucleosomal structure of the episomal c-*myc* was also studied after SoB induction. No differences in the overall nucleosomal patterns of nucleosomes could be detected in untreated and butyrate-treated KH375 cells (Fig. 3D, H, and M) as well as in MA76 and RF266C3 cells (data not shown). However, a new sensitivity to MNase digestion emerged between nucleosomes 3 and 4 after butyrate treatment of KH375 cells (Fig. 3M, lane 2, arrowhead). Taken together, DNase I and MNase analyses of episomal c-*myc* chromatin unambiguously show an increased accessibility in the region upstream of P2 of episomal c-*myc* in RF266C3 and KH375 cells compared to MA76 cells.

Detection of transcription bubbles proximal to the P2 promoter on episomal c-*myc* genes. Interestingly, as in KH375 and RF266C3 cells, the region 70 to 100 bp downstream of the P2 cap site in MA76 cells showed hypersensitivity to MNase (Fig. 3O). Since this region has been shown to be occupied by paused polymerases in RF266C3 cells (67), we next examined whether paused polymerase complexes were also detectable at the P2 promoter on the episomal c-*myc* construct in MA76 cells. To detect stalled complexes, we performed in vivo footprinting experiments based on ligation-mediated PCR (39) by using the single-strand-specific probe $KMnO_4$, which is highly reactive to unpaired T residues in transcription bubbles of paused pol II (27, 77).

Nuclei as well as naked cellular DNA of RF266C3 and MA76 cells were exposed to $KMnO_4$. The patterns of reactive T residues of naked DNA and of DNA derived from nuclei were compared. This analysis revealed hypersensitive T residues in the noncoding strand of c-*myc* DNA of RF266C3 nuclei in a promoter-proximal region between positions +1 and +40 relative to the P2 transcription start site (Fig. 4A, lane 3). For DNA derived from MA76 nuclei, the pattern of hypersensitive T residues downstream of position +1 was almost identical (lane 5). Thus, despite different chromatin structures, RF266C3 and MA76 cells revealed similar $KMnO_4$ sensitivity patterns downstream of the P2 promoter. The pattern observed for episomal c-*myc* in this study is highly similar to the pattern previously described for chromosomal c-*myc* (27). We also monitored $KMnO_4$ sensitivity in nuclei after a transcription reaction. Assuming that reactive T residues downstream of P2 are indicative of transcription bubbles of paused pol II, release of polymerases should result in the loss of $KMnO_4$ hypersensitivity. Indeed, this was the case: the hypersensitivity of T residues at positions +1 to +40 became markedly reduced when a run-on reaction was performed in the presence of all NTPs (Fig. 4A, lanes 4 and 6). When the intensities of bands in both situations (with and without NTPs) were quantitatively compared by phosphorimager analysis (Fig. 4B), reduced sensitivity of T residues in the region +1 to +40 could be confirmed, with slight variations for RF266C3 and MA76 nuclei (for example, in the absence of NTPs, T residues at positions +11, +14, and +40 are hyperreactive in RF266C3 nuclei but not in MA76 nuclei). Thus, the presence of NTPs led to a sensitivity pattern comparable to the one observed for naked DNA.

Paused sense and antisense pol II proximal to the P2 promoter. Nuclear run-on experiments were performed to confirm promoter-proximal pausing of pol II on episomal c-*myc* genes. Hybridization of run-on RNAs to short oligonucleotides specific for c-*myc* exon 1 sequences allowed us to refine the resolution of the assay. By using this method, we could previously

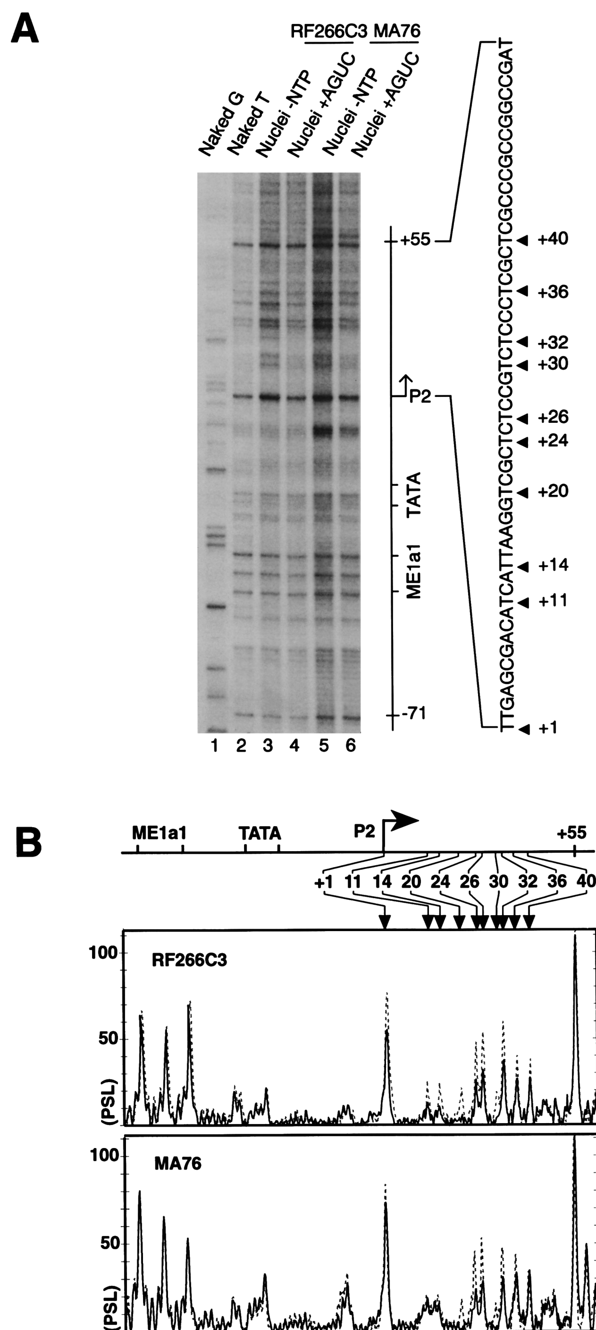

FIG. 4. In vivo footprinting of $KMnO_4$-sensitive sites downstream of the c-*myc* P2 promoter. Non-base-paired T residues at the P2 initiation site and downstream thereof are sensitive to $KMnO_4$ in RF266C3 and MA76 cells and indicative of a paused pol II. (A) The hypersensitivity of T residues from positions +1 to +40 in the template DNA strand in RF266C3 cells (lane 3) and MA76 cells (lane 5) decreases if a nuclear run-on reaction with all four ribonucleotides is performed first (lanes 4 and 6). Cellular naked DNAs treated with dimethyl sulfate (lane 1) and with $KMnO_4$ (lane 2) served as references specific for G and T residues, respectively. Arrowheads indicate hypersensitive T residues. (B) The radioactivity of bands in lanes 3 to 6 was determined with a BAS 1000 phosphorimager (Fuji). Densitometric scans are superimposed to facilitate comparison. The continuous and dashed lines correspond to the sensitivities of T residues before and after, respectively, a nuclear run-on reaction.

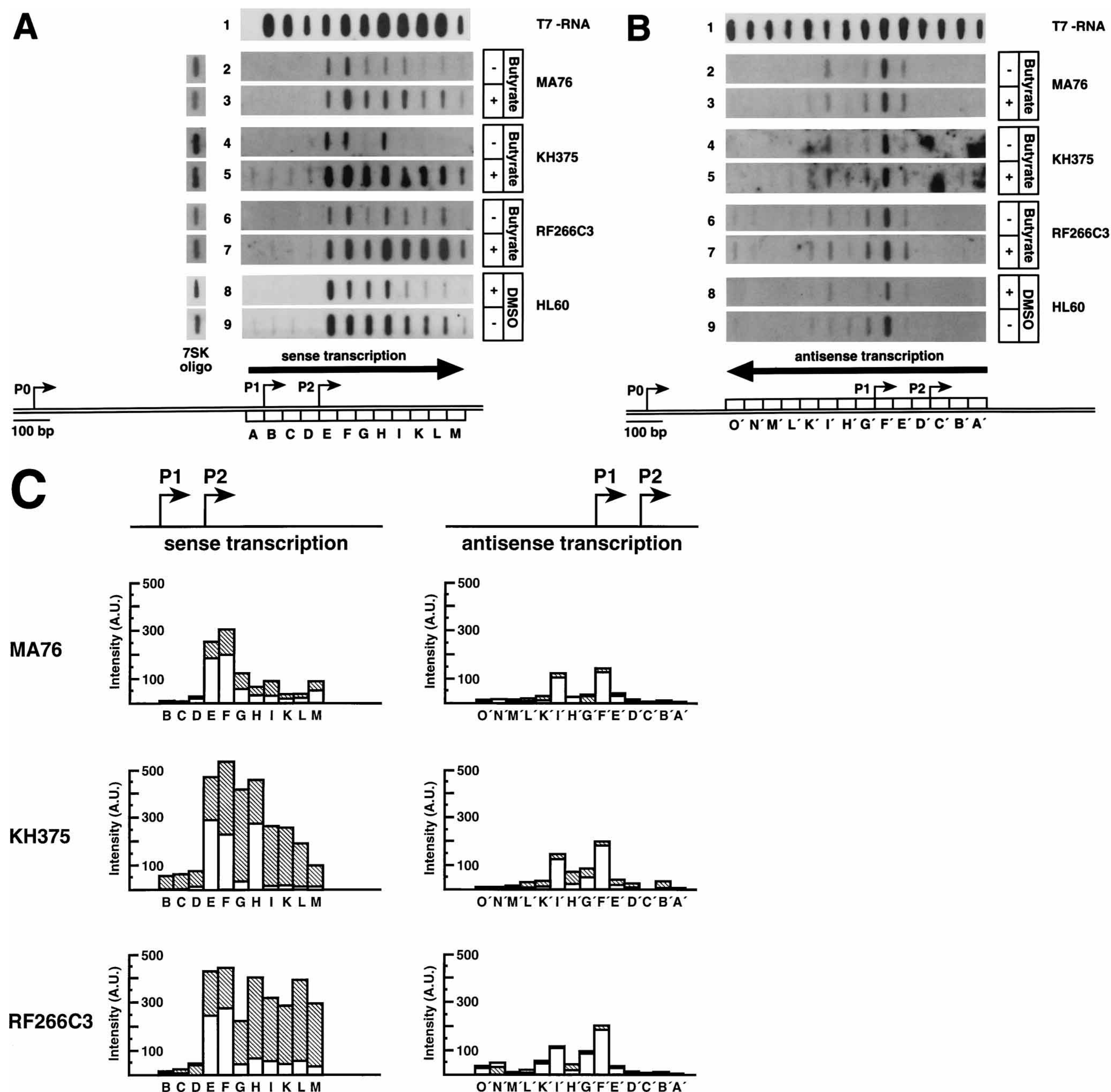

FIG. 5. Pol II distribution within promoter-proximal sequences of c-*myc* genes. Nuclei of untreated (−) or SoB-treated (+) MA76, KH375, and RF266C3 cells and of DMSO-treated (+) or untreated (−) HL60 cells were isolated. Nuclear run-on reactions were performed in the presence of [α-^{32}P]CTP. After the run-on reactions, labeled RNA was isolated and hybridized to membrane-fixed oligonucleotides. Oligonucleotides A to M correspond to the antisense strand of c-*myc* exon 1 with the promoters P1 and P2. Oligonucleotides A′ to O′ correspond to the sense strand of the 5′ half of c-*myc* exon 1 and sequences upstream thereof. Oligonucleotides were used to probe for transcription of c-*myc* in sense (A) and antisense (B) directions. 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 and are shown in lanes 1 of panels A and B. The 7SK oligonucleotide served as a pol III transcription probe. (C) Run-on transcription signals upstream and downstream of the P2 promoter were measured with a BAS 1000 phosphorimager (Fuji). The transcriptional activities for the different oligonucleotides of one representative experiment were determined relative to the corresponding signals of the T7 RNA polymerase and are shown as arbitrary units (A.U.) of intensity. The left and right graphs display quantitated transcriptional activities in sense and antisense directions of untreated (blank columns) and SoB-treated (shaded columns) MA76, KH375, and RF266C3 cells.

show that paused polymerases become transcriptionally activated in nuclear run-on reactions and transcribe a short piece of c-*myc* RNA in HL60 cells (66) and in RF266C3 cells (67). The region transcribed after activation of these polymerases extends to ~100 bp downstream of the c-*myc* P2 promoter.

Nuclei of MA76, KH375, and RF266C3 cells were subjected to run-on reactions; labeled RNAs were purified and hybridized to membrane-bound oligonucleotides A to M, complementary to the entire c-*myc* exon 1 (Fig. 5A). The strong signals seen for oligonucleotides E and F are indicative of paused pol II which becomes activated in the run-on reaction. The signal strengths for both oligonucleotides were almost

identical in the three cell lines, indicating similar amounts of paused pol II (Fig. 5A, lanes 2, 4, and 6). Treatment of KH375 and RF266C3 cells with SoB strongly increased the intensities of signals for oligonucleotides G to M (lanes 5 and 7), while in butyrate-treated MA76 cells, only a minor upregulation of these signals was observed (lane 3). Thus, SoB induced a much higher processivity of pol II in KH375 and RF266C3 cells than in MA76 cells. As control, nuclei of HL60 cells were included (Fig. 5A, lanes 8 and 9). Treatment of HL60 cells with DMSO results in a rapid downregulation of c-*myc* caused by a block to pol II RNA elongation at the P2 promoter (27, 66). The transcription signals obtained for MA76, KH375, and RF266C3 cells were quantitatively evaluated by comparison with signals obtained by a homogeneously labeled c-*myc* exon 1 RNA transcribed by T7 RNA polymerase (Fig. 5A, lane 1). The quantitation revealed an up to eightfold increase of signals for oligonucleotides G to M in butyrate-treated KH375 and RF266C3 cells (Fig. 5C, left graphs). For butyrate-treated MA76 cells, only an up to twofold increase of transcription signals was observed.

Previous analyses of HL60 cells have demonstrated pol II-specific antisense transcription in the c-*myc* gene upstream of exon 1 at a level comparable to that of sense transcription (7). We examined whether this antisense transcription could be delineated more precisely by the nuclear run-on assay. To this end, run-on RNAs of the various cell lines used in this study were hybridized to oligonucleotides A′ to O′, corresponding to a part of sense-strand c-*myc* exon 1 and sequences upstream thereof. A strong signal for oligonucleotide F′ was detected with all run-on RNAs examined (Fig. 5B). Different treatment of cells did not affect the signal for oligonucleotide F′ except for HL60 cells, where DMSO led to a slight decrease of the run-on signal. Again, signals for antisense transcription were compared to those for a homogeneously labeled T7 RNA and quantitated (Fig. 5C, right graphs). The strong signal for oligonucleotide F′ might stem from paused pol II initiated at the P2 promoter in the antisense direction.

DISCUSSION

We have characterized episomal c-*myc* constructs stably assembled as chromatin in different human B cells. The chromatin structure of episomal c-*myc* in KH375 and RF266C3 cells showed a pattern of HSs which was previously described as characteristic of an expressed chromosomal c-*myc* gene (12, 63, 64). In both cell lines, the high sensitivity of HSs III_1 to III_3 to DNase I in the c-*myc* promoter correlated with high sensitivity to MNase, demonstrating the absence of nucleosomes 3 and 4 on almost all episomal constructs. Additionally, a region of ~180 bp centered around the RNA cap site of the P2 promoter was devoid of a detectable nucleosomal structure. In contrast to KH375 and RF266C3 cells, HSs III_1 to III_3 in MA76 cells displayed low sensitivity to DNase I, consistent with clearly detectable nucleosomes 3 and 4. However, similar to KH375 and RF266C3 cells, the region around the P2 cap site in MA76 cells showed hypersensitivity to MNase. Thus, the nucleosomal structure of the episomal c-*myc* in KH375 and RF266C3 cells resembled the structure of an active chromosomal c-*myc* in undifferentiated HL60 cells, while the structure in MA76 cells resembled chromatin of differentiated HL60 cells (53). In MA76 and differentiated HL60 cells, the P2 cap site is still hypersensitive to MNase, indicating that this region is in an open chromatin configuration even if sequences further upstream are occupied by nucleosomes (53).

We and others have previously shown that the P2 promoter of the chromosomal c-*myc* in HL60 cells and of the episomal c-*myc* in RF266C3 cells is repressed by pausing of pol II (27, 66, 67). In vivo footprinting experiments revealed that $KMnO_4$-hypersensitive T residues were found at virtually identical positions in RF266C3 and MA76 cells. Hypersensitive T residues are indicative of a transcription bubble and extended from positions +1 to +40 downstream of P2. These data are in good correlation with $KMnO_4$ footprints showing a transcription bubble in the same region of the chromosomal c-*myc* in HL60 cells (27). Similar to what has been described for HL60 cells, the hypersensitivity of T residues in RF266C3 and MA76 cells decreased when a transcription reaction was performed before $KMnO_4$ treatment. This result strengthens the notion that hypersensitive T residues in MA76 cells reflected the transcription bubble of a paused pol II which was released after addition of NTPs. Pausing of pol II in MA76 cells was also confirmed when nuclear run-on transcripts were labeled. The strength of transcription signals for oligonucleotides E and F in MA76 cells was in the same range as that of signals in KH375, RF266C3, and HL60 cells. All cell lines used in the run-on experiment have comparable numbers of c-*myc* copies. Thus, even though the episomal constructs have established different nucleosomal structures upstream of the P2 promoter in different cell lines, all constructs showed similar extents of paused pol II at the P2 promoter. The finding that pol II can bind efficiently to the P2 promoter in MA76 cells in the presence of nucleosomes 3 and 4 suggests that sequences of the P2 core promoter extending from −70 to +50 might be sufficient to bind pol II not only in vitro and in transient transfection experiments (20, 43) but also when c-*myc* is stably transfected and assembled in chromatin. Within this region of the c-*myc* promoter, the ME1a1, ME1a2, and E2F transcription factor binding sites have been found (4, 20, 38, 71). All of these elements have been shown to contribute to transcriptional activation of c-*myc*. If and how these sites are involved in opening of chromatin and binding of pol II to the P2 promoter remain to be analyzed.

Promoter-proximal pausing of pol II and *cis* elements involved in this regulation have been studied in detail for the *Drosophila hsp70* promoter. Like the c-*myc* gene (78), pol II can pause at variable positions proximal to the transcription start site of the *hsp70* gene (55). The transcription of the gene is activated by the heat shock transcription factor HSF. Upon heat shock, HSF molecules form trimers and bind cooperatively to conserved sequence elements (HSEs) (for a review, see reference 31). Once bound to the array of several HSEs in the *hsp70* promoter region, HSF rapidly stimulates transcription (44). In vitro experiments have shown that HSF binding is strongly inhibited by the assembly of HSEs into nucleosomes (70). In vivo, the HSEs of the *hsp70* gene are cleared of nucleosomes and readily accessible to HSF. Recent studies of the *hsp70* gene indicated that access of HSF to HSEs in vivo depends critically on the promoter architecture defined by GAGA factor, the general transcription factor TFIID, and pol II binding sites (62). As for the *hsp70* promoter, a preset architecture of the c-*myc* P2 promoter could be necessary for the successful induction of the c-*myc* gene. This preset situation might be almost fulfilled for the episomal c-*myc* in KH375 and RF266C3 cells. In these cells, the P2 promoter not only harbors a paused pol II but also has a disruption of two nucleosomes in the further upstream region. Whether disruption of the two nucleosomes allows the binding of factors like HSF to the c-*myc* promoter region remains to be demonstrated. Strikingly, in MA76 cells, these two nucleosomes were clearly detectable in the promoter region, giving a tentative explanation for the failure of SoB to induce the episomal c-*myc* in these cells.

The c-*myc* and *hsp70* promoters differ in several aspects from other inducible promoters. For example, the mouse mammary tumor virus promoter has been shown to be covered by a repressive nucleosome at the transcription start site. Upon induction by hormone, a nucleosome further upstream of this nucleosome is disrupted by the glucocorticoid receptor or the progesterone receptor before binding of the transcription machinery (30, 40, 41, 48, 56). In this case, disruption of nucleosomes further upstream of the transcription start site is required before the transcription machinery is established at the promoter.

When we started these studies, it was not clear whether the κ3′E contributed to the inducibility of c-*myc* in RF266C3 cells. However, the enhancerless construct in KH375 cells showed a c-*myc* chromatin structure and inducibility similar to that in RF266C3 cells. This finding strongly argues that the Raji cell background is responsible for the inducibility of the episomal constructs. One can speculate that in Raji cells, cellular factors bind to the c-*myc* upstream region and prevent the establishment of a regular nucleosomal structure as is seen in MA76 cells. At the same time, these factors may transfer the promoter in a preset stage that is inducible by SoB.

The mode of action of SoB on gene expression is unclear. SoB is a potent inhibitor of deacetylases, and butyrate treatment of cells leads to hyperacetylation of histones (57). Acetylases have recently come into the focus as transcriptional regulators and found to be part of the basal transcription machinery. The p300/CBP protein and the TATA box-binding protein-associated factor $TAF_{II}250$ are both acetyltransferases (37, 45). Whether p300/CBP and $TAF_{II}250$ are involved in activation of the episomal c-*myc* by SoB and whether acetylation positively affects processivity of paused pol II remain to be analyzed. It also needs to be demonstrated at which level TGF-β1 interferes with activation of the episomal c-*myc* by SoB.

While the sense polymerases in KH375, RF266C3, and HL60 cells were able to transcribe c-*myc* productively, antisense polymerases could not be induced for processive transcription. The pause sites of sense and antisense polymerases had similar distances to the TATA box of the P2 promoter. Thus, the same region in the P2 promoter may serve as an entry site for sense as well as antisense polymerases. Entry in either or both directions appears to occur with similar frequency at P2, since run-on signals on oligonucleotide F′ indicative of antisense transcription were as strong as signals generated by sense polymerases. Antisense transcription in MA76 cells indicates that promoter entry of antisense polymerases seems to be unaffected by the presence or absence of nucleosomes 3 and 4 upstream of P2. However, it is unclear whether sense and antisense polymerases can pause on the same template and why these polymerases respond differently to SoB.

Our results demonstrate major differences in the upstream chromatin and in the inducibility of episomal c-*myc* genes carrying transcriptionally engaged pol II. We conclude from our data that upstream chromatin opening of the c-*myc* promoter appears to be one necessary step for the transition of the gene into an inducible activation stage. Whether this step is temporally discernible from binding of polymerases to the promoter in sense and antisense directions and whether promoter entry of pol II always precedes upstream chromatin opening remain to be delineated.

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