The block to transcriptional elongation within the human c-*myc* gene is determined in the promoter–proximal region

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A conditional block to transcriptional elongation is an important mechanism for regulating c-*myc* gene expression. This elongation block within the first c-*myc* exon was defined originally in mammalian cells by nuclear run-on transcription analyses. Subsequent oocyte injection and in vitro transcription analyses suggested that sequences near the end of the first c-*myc* exon are sites of attenuation and/or premature termination. We report here that the mapping of single stranded DNA in vivo with potassium permanganate ($KMnO_4$) and nuclear run-on transcription assays reveal that polymerase is paused near position +30 relative to the major c-*myc* transcription initiation site. Deletion of 350 bp, including the sites of 3′-end formation and intrinsic termination defined in oocyte injection and in vitro transcription assays does not affect the pausing of polymerase in the promoter–proximal region. In addition, sequences upstream of +47 are sufficient to confer the promoter–proximal pausing of polymerases and to generate the polarity of transcription farther downstream. Thus, the promoter–proximal pausing of RNA polymerase II complexes accounts for the block to elongation within the c-*myc* gene in mammalian cells. We speculate that modification of polymerase complexes at the promoter–proximal pause site may determine whether polymerases can read through intrinsic sites of termination farther downstream.

[*Key words*: Transcriptional elongation; attenuation/termination; RNA polymerase II pausing; human c-*myc* gene; potassium permanganate ($KMnO_4$)]

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Although control at the level of transcriptional elongation is a well-established mode of gene regulation in prokaryotes (for review, see Yanofsky 1988), only recently has this mechanism been recognized in eukaryotic cells (for review, see Spencer and Groudine 1990). Viral transcription units, including SV40, adenovirus, and the murine minute virus provided the first examples of premature termination or attenuation in eukaryotes (Hay et al. 1982; Ben-Aser and Aloni 1984; Maderious and Chen-Kiang 1984). Control at the level of elongation has also been observed in several eukaryotic cellular genes, including c-*myc*, c-*myb*, c-*fos*, ADA, l-*myc*, and histone 3.3, and in the human retroviruses HIV-1 and HIV-2 (Spencer and Groudine 1990). For example, the reduction in the steady-state c-*myc* RNA observed during differentiation of different cell types was shown to result from a reduction in the number of polymerases that read through sites of termination or pausing within exon 1. This conditional elongation block was demonstrated originally by nuclear run-on transcription assays, in which exon 1 transcription signals were higher than intron 1 signals (Bentley and Groudine 1986; Eick and Bornkamm 1986; Nepveu and Marcu 1986). Although truncated c-*myc* transcripts with 3′ ends within the first exon or intron could not be detected in mammalian cells, such transcripts are found after injection of the human or murine c-*myc* genes into *Xenopus* oocytes (Bentley and Groudine 1988) and in in vitro transcription assays (Kerppola and Kane 1988; London et al. 1991). The 3′ ends of the most prominent of these RNAs map to two T stretches near the exon 1/intron 1 boundary, at positions +371 and +421 from the transcription initiation site of the major c-*myc* promoter (P2). These T-rich sequences are preceded by regions of dyad symmetry that resemble ρ-independent bacterial terminators (Eick and Bornkamm 1986). Cassettes containing the dyad symmetry have been shown to function as sites of 3′-end formation and intrinsic termination when cloned downstream of some heterologous promoters (Bentley and Groudine 1988; London et al 1991). However, it has not been demonstrated formally that the 3′ ends of the truncated c-*myc* RNAs observed in oocytes or in vitro correspond to the sites of the elongation block observed by nuclear run-on transcription assays in mammalian cells.

The transient pausing of RNA polymerase has been suggested to be a prerequisite for termination (or attenuation) (Platt 1986). Therefore, the mapping of single-

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stranded regions within the c-*myc* gene might identify sites where transcriptional elongation is blocked. Potassium permanganate ($KMnO_4$), which oxidizes T residues in single-stranded DNA very rapidly but is slow to react with double-stranded DNA, is a useful probe for non-base-paired regions in DNA (Borowiec et al. 1987). The differential reactivity to $KMnO_4$ has been exploited in a variety of studies in which the in vitro and in vivo formation of open complexes has been analyzed in prokaryotic and eukaryotic promoters (Sasse-Dwight and Gralla 1988 1989 1990; O'Halloran et al. 1989; Zhang and Gralla 1989; Kassavetis et al. 1990; Vos et al. 1991). Recently, Wang et al. (1992) showed that $KMnO_4$ can detect "transcription bubbles" generated in vitro when ternary RNA polymerase II complexes are stalled. In addition, this reagent has been used to detect the pausing of RNA polymerase at the qut site in phage λ (Kainz and Roberts 1992).

In the studies reported below, we combine $KMnO_4$ treatment of cells with the in vivo footprinting method based on ligation-mediated polymerase chain reaction (PCR) (Mueller and Wold 1989) to map single stranded regions within the human c-*myc* gene in vivo. Our analysis reveals that T residues centering at position +30 relative to the major c-*myc* transcription initiation site are single stranded in conformation in both proliferating and differentiated cells, whereas the T residues near the exon 1/intron 1 boundary show no greater reactivity to $KMnO_4$ than control DNA exposed to $KMnO_4$ in vitro. Nuclear run-on transcription assays reveal that RNA polymerase II complexes are paused in this promoter–proximal region and that the $KMnO_4$ sensitivity of the T residue at position +30 is lost when polymerases are released from the promoter–proximal region. Functional assays demonstrate that the promoter–proximal pausing of polymerase is not affected by the deletion of the sequences that program 3′-end formation and termination in oocyte injection and in vitro transcription assays. In addition, c-*myc* sequences upstream of +47 are sufficient to generate the polarity of transcription observed in the run-on assays. We speculate that the modification of polymerase complexes at the promoter–proximal site may determine whether polymerases can read through intrinsic sites of termination farther downstream.

Results

A potential promoter–proximal pause site revealed in vivo by $KMnO_4$

As described in the introductory section, the positions of open promoter complexes and paused RNA polymerases have been determined by the $KMnO_4$-mapping of single-stranded DNA regions of transcription bubbles in in vitro transcription systems and in prokaryotes in vivo. Thus, we determined whether the $KMnO_4$ assay could be extended to map the in vivo position of attenuated/paused polymerases within the first exon of the human c-*myc* gene. We performed the inititial $KMnO_4$ assays in HL60 cells in which the c-*myc* gene is amplified approximately 10-fold (Collins and Groudine 1982; Dalla-Favera et al. 1982; for review, see in Collins 1987). During differentiation of the human HL60 cell line into granulocytes or macrophages, the down-regulation of c-*myc* steady-state RNA occurs in consecutive steps (for review, see Spencer and Groudine 1991). Exposure of HL60 cells to the differentiation-inducing agent retinoic acid (RA) for several hours results in a reduction in the amount of steady-state c-*myc* RNA by an increase in the block to transcription elongation. After extended exposure to RA for >2 days, the frequency of transcription initiation is reduced, and very little or no c-*myc* transcription initiation is detected when RA treatment is continued for 7 days.

Proliferating HL60 cells and cells induced to differentiate for 2 and 7 days were exposed to $KMnO_4$, and the modified DNA was used in the in vivo footprinting assay (see Materials and methods). The pattern of $KMnO_4$ sensitivity observed in vivo was also compared with that obtained after exposure of purified genomic DNA to $KMnO_4$ in vitro. Surprisingly, comparison of the patterns of $KMnO_4$ sensitivity in the region of the exon 1/intron 1 boundary (Fig. 1) revealed only minor differences among the in vitro-modified DNA (T lane) and DNA from proliferating (prol lane) and differentiated (RA 48h and RA 7d lanes) cells. None of the observed minor differences fulfilled the requirements of a potential pause site. For example, some sites persisted in cells differentiated for 7 days, at which time c-*myc* transcription is not detectable by nuclear run-on transcription assays (Bentley and Groudine 1986; A. Krumm, unpubl.). In addition, these cells also displayed specific $KMnO_4$ sensitive sites that were not detectable in naked DNA, proliferating cells, or cells differentiated for 48 hr. The significance of these sites in the transcriptionally inert HL60 cells differentiated for 7 days is unclear; such changes may reflect alterations in the topology of the c-*myc* gene associated with changes in the chromatin structure of the locus observed after prolonged exposure of HL60 cells to differentiation agents (Siebenlist et al. 1988).

In vivo footprinting of the entire first exon, however, revealed that a region 30 bp downstream of the c-*myc* P2 transcription initiation site was highly sensitive to $KMnO_4$. As shown in Figure. 2,A and B, the T residue at +30 of the noncoding strand is hypersensitive to $KMnO_4$ in both proliferating cells (prol lanes) and cells induced to differentiate for 1 (RA 24h lanes) or two (RA 48h lanes) days but is not sensitive in the in vitro-modified DNA sample (T lane). This implies that these sequences are in a single-stranded conformation in vivo. The hypersensitivity at this position is dependent on the transcriptional activity of the c-*myc* gene, because RA treatment for 7 days results in complete loss of $KMnO_4$ hypersensitivity at this position (see RA 7d lane). We have also observed that the T residue at +30 is hypersensitive to $KMnO_4$ in HeLa cells, in which c-*myc* transcription is high and the c-*myc* gene is not amplified (data not shown). Analysis of $KMnO_4$ sensitivity of the

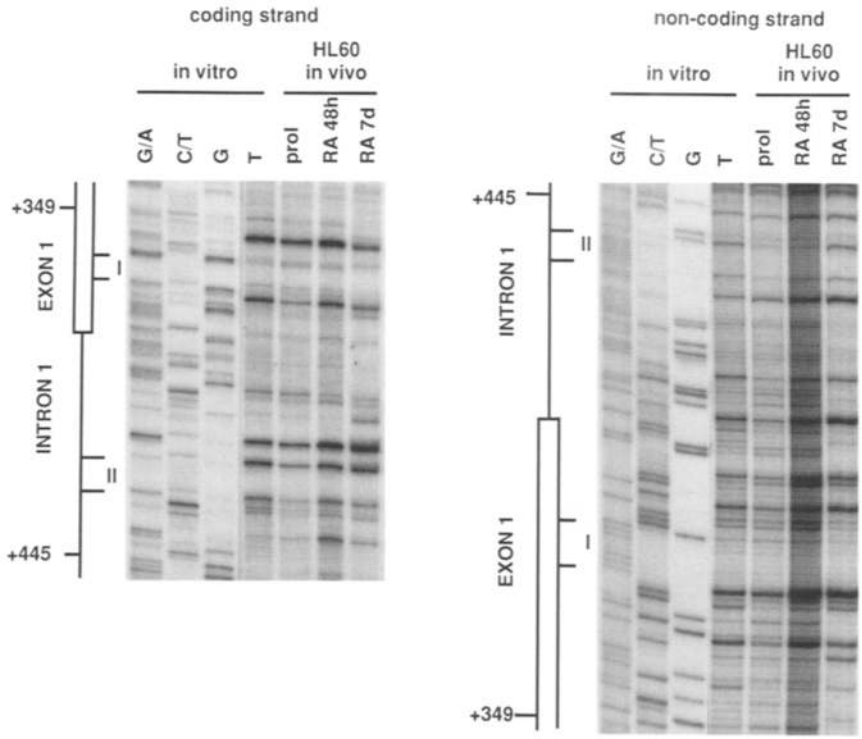

Figure 1. In vivo footprinting of potassium permanganate ($KMnO_4$)-sensitive sites at the c-myc exon 1/intron 1 boundary. The pattern of $KMnO_4$-sensitive residues on both coding and noncoding strands in proliferating (prol) cells and cells differentiated with RA for 48 hr (RA 48h) and 7 days (RA 7d) were compared with genomic DNA treated with $KMnO_4$ in vitro. G/A, G, and C/T sequence ladders are derived from genomic DNA treated in vitro with formic acid (G/A), dimethylsulfate (G), or hydrazine (C/T). No major differences in $KMnO_4$ sensitivity among the in vitro-treated DNA and in vivo-treated proliferating cells and cells differentiated in the presence of RA for 48 hr are detected within positions +349 to +445 relative to the P2 promoter start site. The minor changes in ratios of bands observed in proliferating cells and cells induced for 48 hr vary in different experiments and are not considered significant. Significant differences from the pattern of $KMnO_4$ obtained in vitro are detected only in cells differentiated in the presence of RA for 7 days, at which time c-*myc* transcription initiation is undetectable.

coding strand within the promoter region was uninformative owing to the lack of T residues within the +17 to +55 region (Fig. 2C).

The pattern of $KMnO_4$ sensitivity around position +30 (summarized in Fig. 2C) was not identical in cells at different stages within the HL60 differentiation pathway. In proliferating HL60 cells, T residues at positions +24, +26, +36 and +40 are also hypersensitive to $KMnO_4$, whereas the sensitivity of these residues is diminished in cells induced for 48 hr with RA (Fig. 2B cf. prol and RA 48h lanes). Cells induced for 24 hr (RA 24h lanes) display an intermediate pattern, with a lowered sensitivity to $KMnO_4$ at positions surrounding +30. The differential sensitivity of T residues may reflect differences in RNA polymerase complexes or differences in the nature of pausing/termination reactions (see below).

In addition to the hypersensitivity detected downstream of the P2 transcription initiation site, T residues at positions −40, −44, and −48 were consistently hyposensitive (less reactive) to $KMnO_4$ in cells induced with RA for 48 hr or longer, and a C residue at −37 was hypersensitive in cells induced for 48 hr (Fig. 2B, cf. prol and RA 24h lanes with RA 48h lanes in the ME1a1 region). The basis of these differential sensitivites in the upstream region of the promoter is not clear; however, the region defined by these residues corresponds to the ME1a1 site of protein–DNA interaction in vitro (see Discussion; Asselin et al. 1989; Pyrc et al. 1992).

*The c-*myc *elongation block is due to the promoter–proximal pausing of polymerase*

If the single-stranded region revealed by the $KMnO_4$ assay is the result of the pausing of polymerase II complexes downstream of the transcription initiation site, a higher density of polymerase molecules would be expected in this region. Thus, the pausing of ternary complexes in proliferating and differentiated HL60 cells was further analyzed in nuclear run-on assays in which polymerase II continues to elongate in isolated nuclei in the presence of [^{32}P]UTP. The amount of ^{32}P-labeled RNA transcripts hybridizing to membrane-bound, single-stranded DNA is proportional to the density of elongating polymerase II molecules and the U content of the probes (Bentley and Groudine 1986). The location within the c-*myc* gene and U content of the probes used for these studies is shown in Figure 3A. In analyzing the results of each experiment, the signal derived from the hybridization of nascent transcripts to each c-*myc* probe was corrected for the U content of the probes and normalized to the transcription signal of the H2B gene, which provides an internal standard (Fig. 3C, H2B-normalized graph). The relative distribution of polymerases within the c-*myc* regions corresponding to the individual probes is also presented (Fig. 3D, self-corrected graph).

Nuclear run-on transcription assays were performed under low (37.5 to 75 mM KCL) and high (150 mM KCl) salt conditions, and in the presence of Sarkosyl. Both high salt and Sarkosyl have been shown to release polymerase complexes arrested at the *Drosophila hsp70* gene promoter into sites farther downstream (Rougvie and Lis 1988; O'Brien and Lis 1991). Whereas transcription of the c-*myc* gene is low in nuclei of both proliferating and differentiating cells under low salt conditions, a disproportionally strong increase in signal of promoter–proximal sequences at +47 to +169 (NS probe) is observed

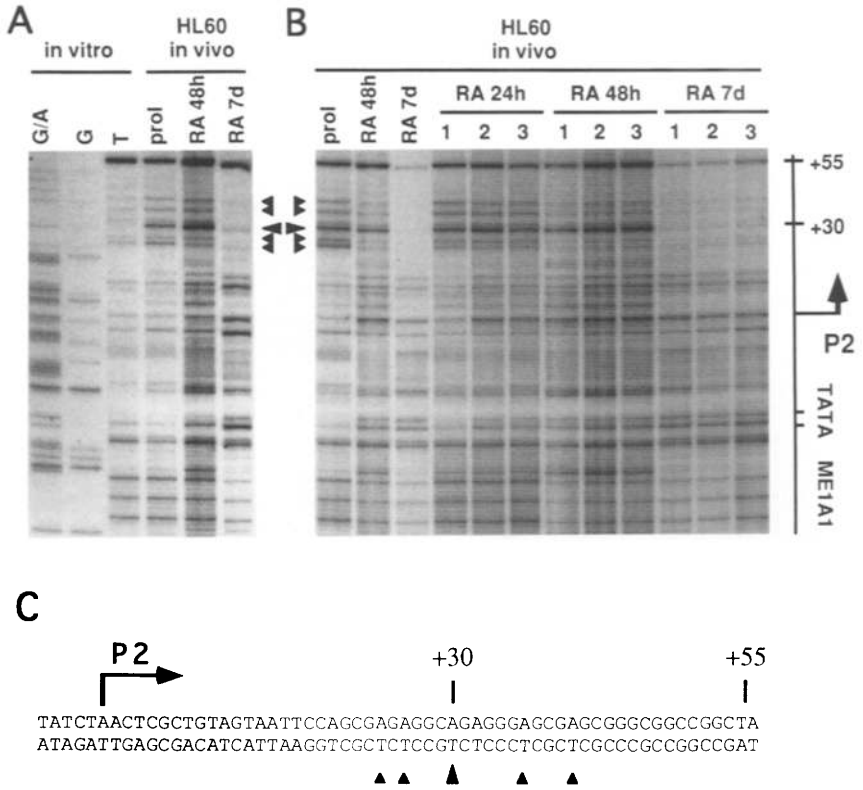

Figure 2. Mapping of potential transcriptional pause sites near the P2 promoter in vivo. Non-base-paired T residues at +30 downstream of the P2 initiation site are sensitive to $KMnO_4$ in cells containing a transcriptionally active c-*myc* gene. (*A*) The in vivo $KMnO_4$ sensitivities of T residues in the template DNA strand of proliferating (prol) HL60 cells and cells differentiated with RA for 48 hr (RA 48h) and 7 days (RA 7d) were compared with DNA treated with $KMnO_4$ in vitro (T). Positions of $KMnO_4$ modifications were determined by comparison to a sequence ladder generated on in vitro-modified, genomic DNA cleaved at G and A residues (G/A), and G (G) residues with formic acid and dimethylsulfate, respectively. Solid arrowheads indicate base residues differentially sensitive in vivo compared with in vitro. (*B*) A separate experiment similar to *A*, except that the in vivo-footprinted DNA from cells induced to differentiate with RA for 24 hr (RA 24h) and different times of exposure to $KMnO_4$ are included. Numbers *1*, *2*, and *3* refer to different exposure times (1, 2, and 4 min) of HL60 cells to $KMnO_4$. (*C*) The DNA sequence and $KMnO_4$-sensitive T residues within this region are shown.

when the run-on assays are performed in high salt (Fig. 3). These results suggest that RNA polymerase II complexes pause in the promoter–proximal region in vivo in both proliferating cells and cells differentiated for 48 hr, and that these polymerases are released to elongate in vitro by high salt. This effect cannot be attributed simply to the removal of "roadblocks" such as histones, because 150 mM KCl is not sufficient to remove histones from DNA (Burton et al. 1978). Run-on experiments were also performed with nuclei of HL60 cells differentiated for 7 days; however, these experiments were not interpretable owing to the low level of transcription in these nuclei (data not shown).

The nuclear run-on assays also reveal that polymerase complexes in differentiated cells are not as processive as those in proliferating cells. Under low salt conditions, the polymerase density does not vary over different regions of exon 1 and intron 1 in nuclei of proliferating cells, whereas in differentiated cells a signal approximatly fivefold higher is observed in the promoter–proximal NS fragment. The high salt nuclear run-on experiments also reveal a difference in the density of transcribing polymerases downstream of +169 in proliferating and differentiated cells (Fig. 3D, cf. the ratios of NS/RS in proliferating and differentiated cells). These results confirm previous observations (Bentley and Groudine 1986; Eick and Bornkamm 1986) that polymerase II transcription complexes in differentiating cells are not as processive as those in proliferating cells. However, in contrast to previous reports, our results suggest that the c-*myc* elongation block is determined within the region upstream of +169, rather than at the end of exon 1. Thus, promoter–proximal sequences appear to be important mediators of the down-regulation of c-*myc* steady-state RNA levels in differentiating HL60 cells. Nuclear run-on analyses of c-*myc* transcription in the pluripotent K562 cell line, which contains one copy of the c-*myc* gene and can be induced to cease proliferation in response to the differentiation agent HMBA, also reveal that polymerase complexes in proliferating cells are more processive than those in induced cells, and RNA polymerase complexes stalled in the promoter–proximal region can be induced to elongate under conditions of high salt (data not shown). Thus, in two different cell lines, the pausing of polymerase in the promoter–proximal region of the c-*myc* gene accounts fully for the high density of polymerase in exon 1 reported previously.

The addition of 0.6% Sarkosyl to nuclear run-on assays performed in low (Fig. 3) or high salt (data not shown) also revealed a disproportionately high increase in signal in the promoter–proximal NS fragment in both proliferating and differentiated cells. As described above, these results suggest that polymerase complexes are paused in the promoter–proximal region in vivo, and that these complexes are released to elongate in vitro by Sarkosyl. However, as shown in other systems (O'Brien and Lis 1991), ternary complexes farther downstream are not as efficient in resuming elongation in the presence of Sarkosyl, as indicated by the low RS signal compared with the NS signal in the run-on transcription assays performed with nuclei of proliferating cells (Fig. 3D). This observation suggests that the promoter–proximal

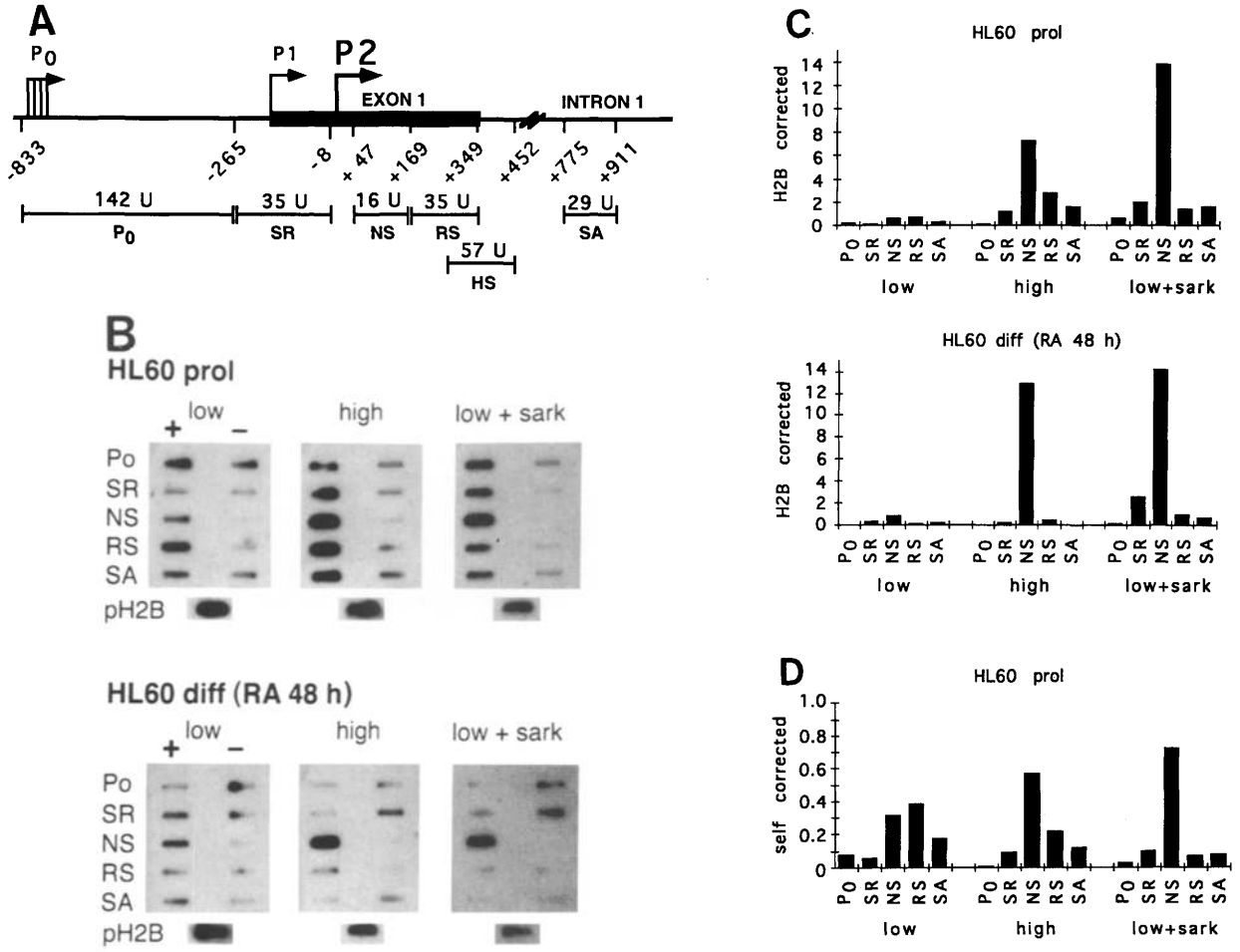

Figure 3. Detection of paused polymerases in nuclear run-on transcription assays. (*A*) Positions of single-stranded probes used to detect RNA synthesized in nuclear run-ons. (P0, P1, P2) Positions of transcription initiation in the human c-*myc* gene. The uridine (U) content of transcripts corresponding to individual fragments is shown above each probe. In nuclear run-on experiments with transfected L cells (Fig. 4), an additional probe (HS) was used. (*B*) Run-on assay with nuclei of proliferating (prol) and differentiating (diff RA 48 h) HL60 cells under low (50 mM KCl) and high (150 mM KCl) salt conditions and in the presence of 0.6% Sarkosyl. Pulse-labeled RNA was hybridized to single-stranded DNA regions containing the antisense (+) and sense (−) c-*myc* sequences. In control experiments, α-amanitin was used to verify that the signal obtained under these conditions is the result of polymerase II transcription (Bentley and Groudine 1986; data not shown). (pH2B) A probe detecting transcription of the H2B gene. (*C*),(*D*) Quantitation of the nuclear run-on shown in *B*. Signals were scanned and quantified using a PhosphorImager system (Molecular Dynamics). Background signals were counted separately and subtracted. Values were machine counts of radioactivity bound to immobilized c-*myc* DNA, corrected for the U content of each probe, and normalized to the signals obtained with the H2B-probe (H2B-corrected graph; *C*), or to the sum of signal obtained with the P0, SR, NS, RS, and SA probes (self-corrected graph; *D*).

polymerase complexes are biochemically distinct from those that have elongated farther into the gene. The use of Sarkosyl in various in vitro transcription assays has also provided evidence for distinct promoter proximal and downstream ternary complexes (Linn and Luse 1991; Kephart et al. 1992).

Polymerase complexes paused in the promoter–proximal region are initiated at the P2 promoter

Recently, *Xenopus* oocyte injection experiments have revealed that 3′ ends of transcripts initiated at the upstream P1 promoter can be formed within the P2 promoter (Wright et al. 1991; Meulia et al. 1992; Roberts et al. 1992). Thus, the elongation of polymerase II complexes initiated at the P1 promoter may be "blocked" within the P2 promoter in mammalian cells. Consequently, the observed increase in signal within the promoter–proximal NS fragment after high salt or Sarkosyl treatment of nuclei could be the result of the release of these P1 transcription complexes. To investigate this possibility, we performed nuclear run-on analyses of

mouse L cells containing a transfected wild-type human c-*myc* gene (BS2) or a mutated human c-*myc* gene (ΔP1) in which the P1 promoter is deleted and P1 transcription is abolished (Spencer et al. 1990). As shown in Figure 4, the same pattern of c-*myc* transcription was observed in nuclei of transfected polyclones containing the wild-type or ΔP1 c-*myc* gene. Similar to HL60 cells, stalled polymerases were released into the NS fragment when the salt concentration was raised to 150 mM KCl or 0.6% Sarkosyl was added. These results suggest that the high salt/Sarkosyl-induced release of polymerases into the P2 promoter–proximal region is the result of P2-initiated transcription complexes, rather than P1-initiated polymerases. It should be noted that in this experiment, the NS signal in the transfected human c-*myc* genes is higher than the RS signal under low salt run-on conditions, similar to the results obtained in the differentiated HL60 and K562 cells. In separate experiments, we have observed that the NS/RS ratio varies under low salt conditions in transfected L-cell pools but that the release of polymerases into the NS fragment by high salt or Sarkosyl is invariant (data not shown).

Sequences at the exon/intron boundary are dispensable for the block to elongation

The nuclear run-on analyses presented above suggest that the promoter–proximal pausing of polymerase complexes accounts fully for the polarity of transcription within the first c-*myc* exon reported previously. However, oocyte injection and in vitro transcription analyses have revealed that sequences within the c-*myc* exon 1/intron 1 boundary region are sufficient to program 3′-end formation and termination, respectively, in c-*myc* and chimeric genes (for review, see Spencer and Groudine 1990,1991; also see Introductory section). To determine whether the intron 1/exon 1 sequences defined as necessary for 3′-end formation and termination in the oocyte injection and in vitro transcription assays were essential for the block to elongation observed in mammalian cells, a 343-bp deletion from position +269 to +611 was made in the wild-type human c-*myc* template (Fig. 5A). This construct (pBS2Δ343) was transfected into mouse L cells, stable polyclones were isolated, and the pattern of c-*myc* transcription was determined by nuclear run-on analyses (Fig. 5B). Similar to the wild-type c-*myc* gene, transcription of the deletion mutant under low salt conditions is low and a disproportionate increase in the promoter–proximal signal is observed when the run-on analyses are performed under high salt conditions. Thus, sequences within the exon 1/intron 1 boundary region do not contribute to the c-*myc* block to elongation defined by nuclear run-on analyses in mammalian cells.

c-myc *sequences upstream of position +47 are sufficient to confer the block to elongation*

The $KMnO_4$ sensitivity in both proliferating and differentiating HL60 cells centers around +30 (Fig. 2), and the highest rate of transcription in nuclear run-on assays (Figs. 3–5) is observed just downstream (+47 to +169). In combination, these data suggest that the sequences upstream of +47 may confer the pausing and Sarkosyl/high salt release of polymerase. To test this possibility, all c-*myc* sequences downstream of +47 were replaced

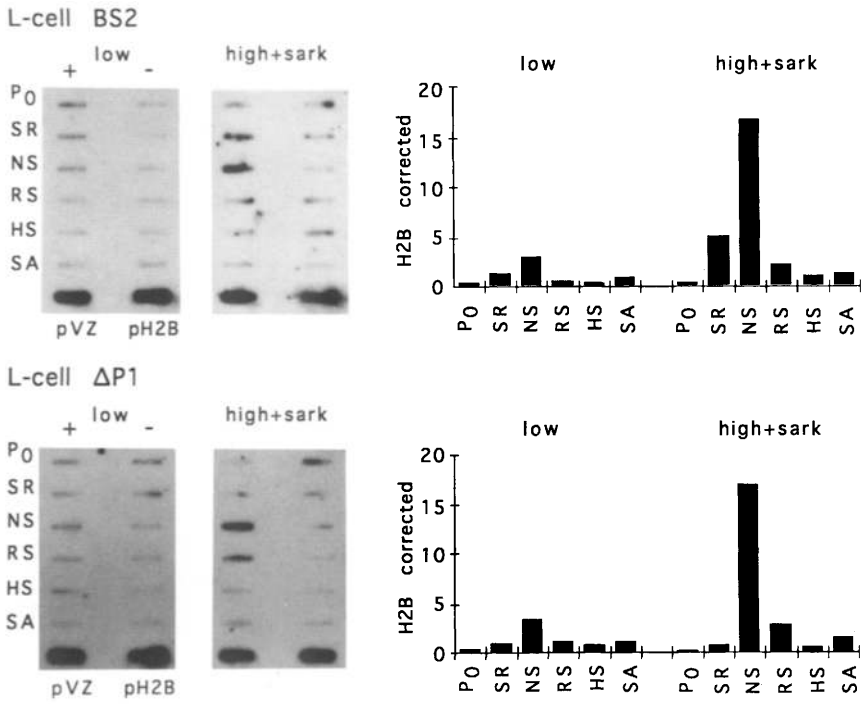

Figure 4. Paused/released polymerases are initiated at the P2 promoter. Mouse L cells, stably transfected with a wild-type c-*myc* gene (BS2) and a mutant c-*myc* gene (ΔP1) containing a 30-bp deletion within the P1 promoter (Spencer et al. 1990) were analyzed in nuclear run-on assays. Control experiments have shown that endogeneous mouse *myc* transcripts do not cross-hybridize to exon 1 and intron 1 sequences of the human c-*myc* gene (data not shown).

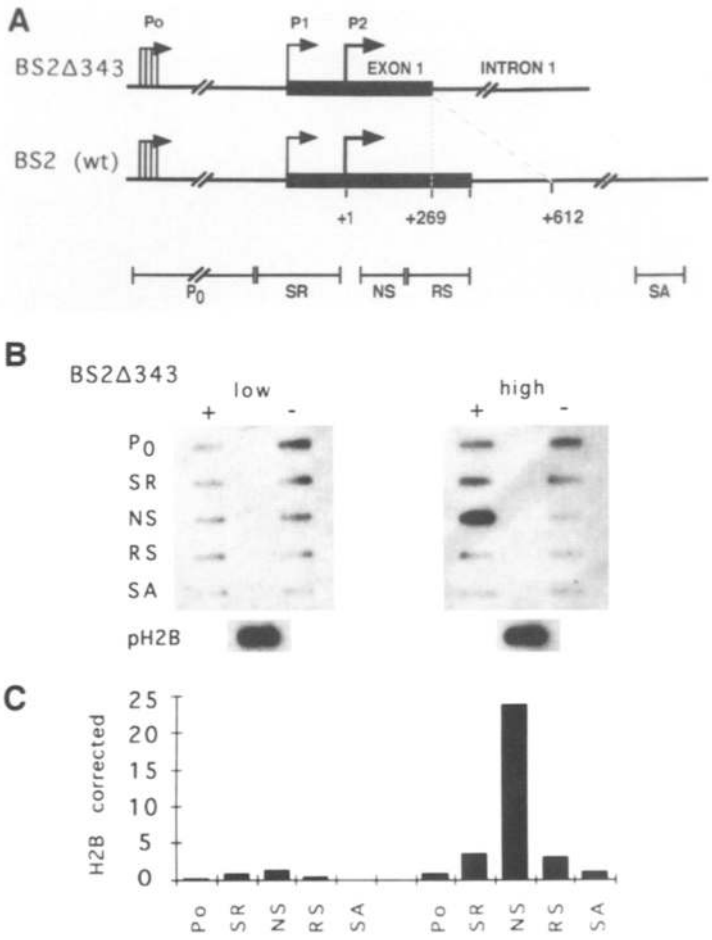

Figure 5. Sequences at the exon 1/intron 1 boundary are dispensable for the c-*myc* block to elongation in mammalian cells. (*A*) Map of deletion mutant BS2Δ343, from which sequences from +269 to +611 relative to the P2 transcription initiation site of the wild-type (wt) c-*myc* clone (BS2) are deleted (see Materials and methods). (*B*) Nuclear run-on transcription assays performed in low (50 mM) and high (150 mM) KCl with nuclei of polyclonal L cells stably transfected with the c-*myc* deletion construct BS2Δ343. The position of single-stranded DNA probes (P0, SR, NS, RS, and SA) detecting sense (+) and antisense (−) transcripts are shown below the map of BS2 in *A*. (pH2B) A probe that detects transcription of the H2B gene. (*C*) Bar graph of transcription signals as quantitated with the PhosphorImager system. Signals were corrected for the U content of each probe. The U content of transcripts hybridizing to the RS probe was calculated as 18, which represents the part of the RS sequence remaining in the BS2Δ343 mutant.

with the bacterial chloramphenicol acetyltransferase (CAT) gene, and this chimeric *myc*/CAT construct was transfected into mouse L cells. A control H1°/CAT construct (generous gift of L. Johnston and H. Eisen, Fred Hutchinson Cancer Center), consisting of the murine histone H1° promoter linked to the same CAT sequences, was also transfected into L cells. In nuclear run-on transcription assays performed under low salt conditions, polymerase density does not vary significantly within the 5′ middle and 3′ CAT probes in either construct (Fig. 6). However, when the nuclear run-on assays are performed in the presence of Sarkosyl, a disproportionately high signal is observed in the promoter–proximal CAT sequences (CAT1) in the *myc*/CAT construct. In contrast, no significant differences in signal are observed within the three CAT probes in nuclei from the L cell pools containing the H1°/CAT gene, under conditions of low salt or Sarkosyl. Therefore, the high density of polymerase in the 5′ CAT sequences observed in the *myc*/CAT gene in nuclear run-on assays performed in the presence of Sarkosyl is not the result of an intrinsic property of the CAT sequences. These results indicate that c-*myc* promoter elements, including the first 47 nucleotides downstream of the transcription initiation site, are sufficient to cause the promoter–proximal pausing and high salt/Sarkosyl release of RNA polymerase II transcription complexes.

Release of polymerase complexes results in the loss of $KMnO_4$ sensitivity at +30

The experiments described above revealed that RNA polymerase II complexes are paused upstream of +169 within the wild-type human c-*myc* gene and that sequences upstream of +47 are sufficient to program the pausing of polymerase complexes. In addition, mapping of $KMnO_4$ sensitive sites within the transcriptionally active c-*myc* gene suggests that the pause site is located at +30. If the release of RNA polymerase II complexes observed in the nuclear run-on assays does, in fact, occur from a pause site at +30, the $KMnO_4$ sensitivity of the T residue at position +30 should be diminished significantly after the release of polymerase. Thus, we monitored the $KMnO_4$ sensitivity of the c-*myc* promoter–proximal region in isolated nuclei before and after the release of polymerase in high salt nuclear run-on conditions. As shown in Figure 7, hypersensitivity of the T residue at position +30 is observed in nuclei isolated from proliferating HL60 cells, when these nuclei are exposed to $KMnO_4$ before the addition of ribonucleotides

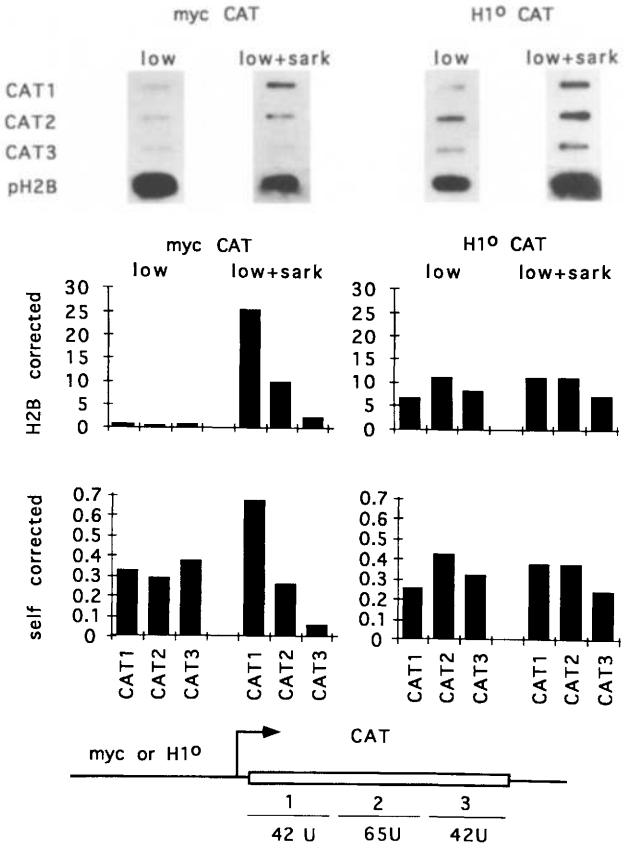

Figure 6. c-*myc* sequences upstream of +47 are sufficient for the promoter–proximal pausing of polymerase. The *myc*/CAT and H1°/CAT constructs containing the human c-*myc* P2 promoter (−2489 to +47 relative to the P2 start site) or the murine histone H1° promoter (−1600 to +108) upstream of 659 bases of the CAT gene coding sequence (see Materials and methods) were transfected independently with a neomycin resistance gene into mouse L cells, and stably transfected polyclones containing either construct were selected in G418. Nuclear run-on assays were performed with nuclei of polyclonal mouse L cells containing either the *myc*/CAT or the H1°/CAT gene, under conditions identical to those described in Fig. 3. The single-stranded probes used to detect sense transcripts within the CAT sequences correspond to positions −49 to +113 (CAT1), +171 to +393 (CAT2), and +514 to +630 (CAT3), relative to the first base of the CAT AUG initiation codon. The relative position and U content (CAT1 = 42, CAT 2 = 65 and CAT 3 = 42) of the CAT probes are shown below the line drawing.

(Fig. 7, −NTP lane). Under these conditions, no significant nascent chain elongation occurs (Stallcup et al. 1978). However, hypersensitivity of the T residue at position +30 is not observed when the nuclei are exposed to $KMnO_4$ 15 min after the addition of ribonucleotides (Fig. 7, +NTP lane)—conditions in which paused polymerases are released from the promoter–proximal region (Figs. 3–5). Thus, the loss of sensitivity of the T residue at position +30 to $KMnO_4$ correlates with the release of polymerase from the promoter–proximal region. These results are consistent with a model in which RNA polymerase II complexes are paused at or near postion +30; and under conditions of high salt (or Sarkosyl), these complexes are released to elongate.

Discussion

A combination of $KMnO_4$ and nuclear run-on analyses suggest that RNA polymerase II complexes are paused within the first 47 bp downstream of the P2 initiation site. Sequences upstream of +47 relative to the P2 initiation site are sufficient to cause the promoter–proximal pausing of polymerase, even when heterologous sequences are cloned downstream of +47. Thus, the block to c-*myc* elongation in mammalian cells described previously is the result of paused polymerase complexes in the P2 promoter proximal region, rather than at the end of exon 1. P2-initiated polymerases in proliferating cells elongate more efficiently downstream of the promoter–proximal region than do polymerase complexes in differentiated cells. In addition, the $KMnO_4$ sensitivity around +30 is distinct in proliferating and 48-hr-differentiated cells. These results (summarized in Fig. 8) are consistent with a model in which the pausing of polymerase at this promoter–proximal site allows modification of the ternary complex, rendering the complex susceptible or resistant to intrinsic termination signals farther downstream. The nuclear run-on experiments presented above suggest that these termination sites are located upstream of +169, because the major change upon differentiation of HL60 cells is the decrease in elongating polymerase complexes in the RS fragment (+169 to +349) relative to the NS fragment (+47 to +169). Although oocyte injection and in vitro transcription assays have shown that a region near the end of exon 1 containing dyad symmetry and two runs of T residues can function as

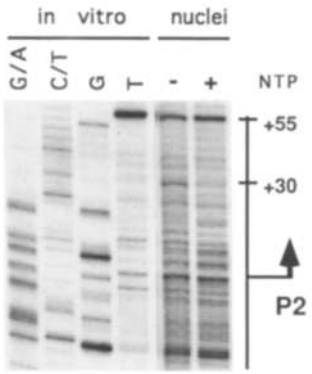

Figure 7. Loss of the $KMnO_4$ sensitivity of the T residue at position +30 under conditions in which polymerases are released from the promoter–proximal region. Proliferating HL60 nuclei were incubated under high salt (150 mM KCl) nuclear run-on conditions. Nuclei were exposed to $KMnO_4$ before (−NTP) and 15 min after (+NTP) the addition of ribonucleoside triphosphates. Genomic DNA exposed to $KMnO_4$ in vitro (T) serves as a control. Positions of residues modified by $KMnO_4$ were determined by comparison to a sequence ladder generated on in vitro-modified genomic DNA cleaved at G and A (G/A), C and T (C/T), and G (G) residues.

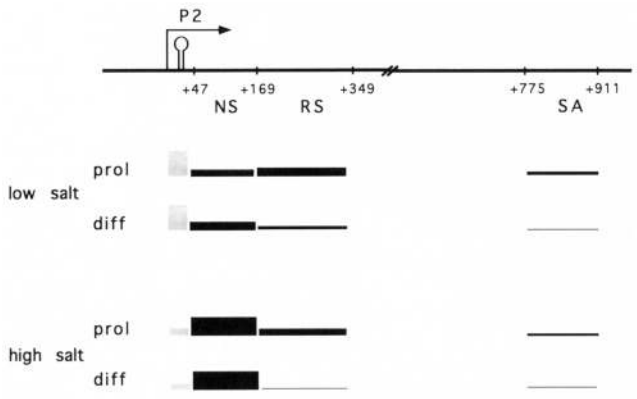

Figure 8. Summary and interpretation of nuclear run-on transcription results. The map coordinates of the various c-*myc* probes and a stem–loop located from +3 to +25 relative to the P2 start site (see Discussion) are shown. Solid bars represent the density of polymerases within different regions downstream of +47 of the c-*myc* gene, as measured in nuclear run-on assays. Pausing of polymerases upstream of +47 is indicated by the stippled areas and is deduced from the different results obtained under conditions of low salt, high salt, and Sarkosyl in HL60 and K562 nuclei and in L cell nuclei containing the BS2Δ343 or the *myc*/CAT fusion gene. Under low salt conditions, the paused promoter–proximal polymerases are not released efficiently into the NS fragment. Under high salt conditions (or in the presence of Sarkosyl), these paused polymerases are released. In addition, there are fewer elongating polymerases downstream of +169 in differentiating compared with proliferating cells. In combination, the nuclear run-on and $KMnO_4$ results (Fig. 2) suggest that the polymerase complexes are paused at approximately +30, and that modification of transcription complexes at this site may be responsible for the differences in elongation efficiencies of polymerase complexes in proliferating and differentiated cells.

efficient termination signals, our deletion analysis demonstrates that this region is dispensable for the c-*myc* elongation block defined by nuclear run-on transcription assays in mammalian cells. Of potential significance, c-*myc* 3′ ends have also been observed upstream of position +169, both in oocyte injection experiments (Bentley and Groudine 1988) and in vitro (London et al. 1991). Whether these additional sites can function to terminate transcription in vivo remains to be determined.

The mapping of $KMnO_4$ sensitive sites in HL60 cells suggests that the polarity of polymerase density observed under high salt/Sarkosyl nuclear run-on conditions results from the release of RNA polymerase II complexes from position +30. Consistent with this, sequences downstream of +47 are not required to generate pausing of polymerase II complexes and the polarity of transcription. In addition, the loss of the $KMnO_4$ sensitivity of the T residue at position +30 in isolated nuclei, after RNA polymerase II complexes are released by the addition of ribonucleotides, strongly suggests that the pause site is located at or near +30. In this context it should be noted that in contrast to the T residue at +30, T residues at positions +1, +2, +11, +14, +15, and +26 of the noncoding strand are not sensitive to $KMnO_4$, suggesting further that polymerase complexes are released from the +30 region, rather than from the promoter itself.

The mechanism of pausing of polymerases in the promoter–proximal region of the human c-*myc* gene is unknown. Recently, in vitro transcription experiments with reconstituted nucleosomal DNA templates revealed that histones can induce or increase pausing of mammalian RNA polymerase II (Izban and Luse 1991). These studies revealed that elongation of RNA polymerase II in vitro through nucleosome-associated DNA is more efficient in the presence of Sarkosyl, which removes histones from DNA, than in the presence of high salt. However, because the release of polymerase complexes in the promoter–proximal region of the c-*myc* gene is similar in high salt and Sarkosyl, it is unlikely that nucleosome positioning is the basis of the pausing of polymerase in the c-*myc* promoter–proximal region.

Analysis of transcription in prokaryotes has suggested that GC-rich regions and stem–loop structures may contribute to polymerase pausing (Levin and Chamberlin 1987). Computer analysis of the c-*myc* P2 promoter–proximal sequence reveals a dyad symmetry capable of forming a 7-bp stem and 9-nucleotide loop structure from position +3 through +25 relative to the P2 transcription initiation site, just upstream of the pause site defined by the $KMnO_4$ sensitivity studies and nuclear run-on analyses (Fig. 8). T1 ribonuclease analysis of a synthetic RNA containing these sequences has verified their ability to form a stem–loop in vitro (A. Krumm, unpubl.). Interestingly, this sequence is completely conserved in the murine c-*myc* gene.

The pausing of polymerase within the first 47 nucleotides downstream of the P2 initiation site may be similar to the pausing of polymerase at the bacteriophage λ qut site at +16, where, in the presence of Q protein, polymerase is modified into a form that reads through termination signals farther downstream (Roberts 1988). Recently, the *E. coli* RNA polymerase pause site at qut has been shown to contain single-stranded regions, as defined by $KMnO_4$ sensitivity, both in vitro and in vivo (Kainz and Roberts 1992).

It is also possible that the down-regulation of c-*myc* expression in differentiating cells is accomplished simply by decreasing the frequency of release of ternary complexes paused 5′ to +47. A similar mechanism has been described for the *hsp70* gene in *Drosophila*, where elements upstream of the transcription initiation site as well as sequences at the +25 pause site contribute to the pausing of RNA polymerase complexes (Lee et al. 1992). The pausing of polymerase at the *hsp70* promoter was shown originally by nuclear run-on transcription and UV cross-linking analyses (Gilmour and Lis 1986; Rougvie and Lis 1988). Recently, this pause site has also been mapped by the identification of $KMnO_4$-sensitive sites in the region of +14 through +42 (Giardina et al., this issue). The length of the observed $KMnO_4$-sensitive region suggests that polymerase II complexes may pause at multiple sites in the *hsp70* promoter–proximal region. Similarly, we observe multiple $KMnO_4$-sensitive T resi-

dues at postion +24 to +40 in the c-*myc* promoter–proximal region, particularly in proliferating cells. These results suggest that the regulation of elongation in the human c-*myc* and *Drosophila hsp70* genes may be through similar mechanisms. Of potential interest, sequences analogous to the GAGA element, which is necessary for the promoter–proximal pausing of polymerase in the *hsp70* promoter, are present upstream of the c-*myc* P2 initiation site. These sequences define the binding site of the ME1a1 protein (Asselin et al. 1989; Pyrc et al. 1992), which has been implicated in the transcription elongation block in the murine c-*myc* gene (Miller et al. 1989). Interestingly, the human equivalent of this sequence shows consistently less modification by $KMnO_4$ in cells induced with RA (Fig. 2). Thus, the change in reactivity of these residues may be the consequence of a change in DNA conformation mediated by DNA-binding proteins or the protection of DNA by such proteins. Although accessibility of base residues to $KMnO_4$ has been thought to be unaffected by DNA–protein interactions (Borowiec et al. 1987), recent results suggest that this type of protection is possible (Giardina et al., this issue).

Our results are also reminiscent of those described for the regulation of human immunodeficiency virus (HIV) transcription, in which elongation-deficient polymerase complexes are modified into more processive complexes in the promoter–proximal region. The generation of processive RNA polymerase complexes in HIV is mediated through the interaction of Tat protein with the TAR RNA stem–loop element that resides from position +1 to +56 (Kao et al. 1987; Berkhout et al. 1989; Laspia et al. 1989; Selby et al. 1989; Toohey and Jones 1989; Ratnasabapathy et al. 1990; Kato et al. 1992; for reviews, see Jones 1989; Cullen 1990; Rosen 1991; Frankel 1992). In addition, nuclear run-on analyses of several *Drosophila* genes have revealed that the promoter–proximal pausing of polymerases is a general phenomenon (Rougvie and Lis 1990). Thus, the modification of ternary complexes at promoter–proximal sites may be an important mechanism in controlling eukaryotic gene expression. Recent in vitro transcription analyses of a number of *Drosophila* genes have shown that RNA polymerase complexes pause and subsequently terminate during the the initial phase of elongation just after transcription initiation, resulting in the generation of short transcripts (Kephart et al. 1992). The transition from this abortive elongation phase to productive elongation appears to be regulated by both positive and negative factors (Marshall and Price 1992). It will be interesting to determine whether these observations can be extended to mammalian genes, including c-*myc*, that are regulated by a conditional elongation block.

Materials and methods

Construction of mutants and M13 clones

The full-length wild-type pBS2 c-*myc* clone and derivative pΔP1 c-*myc* clone from which the P1 TATA box through P1 cap site are deleted have been described previously (Spencer et al. 1990). The p*myc*–CAT construct was made by cloning the 2536-bp *Hin*dIII–*Nae*I c-*myc* promoter fragment (−2489 to +47) of pΔP1 (Spencer et al. 1990) into pBLCAT3 (Luckow and Schutz 1987) containing the bacterial CAT gene and the SV40 polyadenylation region and the small t-antigen region. The plasmid pBLCAT3 was cut with *Xho*I and, after ends were made blunt with T4 polymerase, digested with *Hin*dIII. The 343-bp deletion in pBS2Δ343 was generated in the human c-*myc* *Kpn*I–*Xba*I fragment (from position −1218 to +1019 relative to the P2 transcription initiation site) subcloned into the pBluescript II SK(+) vector, according to the modified exonuclease III-deletion method of Henikoff (1990). The *Rsr*I–*Xba*I fragment containing the deletion was inserted into pBS2 (Spencer et al. 1990). The position and extent of the deletion were determined by sequence analysis.

M13 clones of regions of the c-*myc* gene used as probes for nuclear run-on transcription assays were generated by isolating a 122-bp *Nae*I–*Rsr*I (+47 to +169 relative to the P2 transcription initiation site), a 180-bp *Rsr*I–*Pvu*II fragment (+170 to +349), a 192-bp *Hin*fI fragment (+260 to +452), and a 136-bp *Sac*I–*Acc*II fragment (+775 to +911) and inserting them into M13mp18 and M13mp19 vectors to obtain the NS, RS, HS, and SA sense and antisense probes. The SR probe was constructed by subcloning a *Sma*I–*Eco*RI fragment from pLS13 (Meulia et al. 1992) into M13 vectors.

CAT probes for nuclear run-on assays were constructed by inserting CAT sequences of pCATbasic (Promega) corresponding to −49 to +113 (*Xba*I–*Pvu*II, CAT1), +171 to +393 (*Hae*III, CAT2) and +514 to +630 (*Msc*I–*Sca*I, CAT3) into M13mp18 or M13mp19.

Cell lines and transfections

HL60 cells (for review, see Collins 1987) were maintained in RPMI medium supplemented with 10% fetal calf serum and 2 mM glutamine. Differentiation of HL60 cells was induced by the addition of RA (Sigma) to a final concentration of 10 μM.

L cells were grown on petri dishes in Dulbecco's modified Eagle medium (DMEM) with 10% bovine calf serum. For transfection, 1×10^7 to 2×10^7 cells in DMEM without serum were electroporated in the presence of 15 μg of plasmid DNA linearized with *Eco*RI (pΔP1 and pBS2) or *Aat*II (pΔP1CAT) along with pRSV–*neo* (Gorman et al. 1983). Stably transfected polyclonal lines were established by selection in 1 mg/ml of G418 for 2 weeks.

In vivo footprinting with $KMnO_4$

To probe for $KMnO_4$ sensitive sites within the c-*myc* gene, HL60 cells were harvested and resuspended in medium to a density of 1×10^7 to 2×10^7/ml. $KMnO_4$ was added to a final concentration of 10 mM, and aliquots of cells were taken at 1, 2, and 4 min and added immediately to a lysis buffer containing 70 mM Tris-HCl (pH 7.4), 2 M NaCl, 35 mM EDTA, 3.5% SDS, and 0.7 M β-mercaptoethanol. The $KMnO_4$ treatment of nuclei was conducted as described for whole cells, with the exception that the nuclei were suspended at a concentration of 2×10^8/ml in nuclear run-on buffer (Bentley and Groudine 1986) and exposed to $KMnO_4$ at 37°C in the presence or absence of ribonucleotides. Genomic DNA was purified with phenol–chloroform and precipitated with ethanol at room temperature. In vitro permanganate modification was performed by treatment of up to 20 μg of purified genomic HL60 DNA in H_2O with 10 mM $KMnO_4$ (final concentration) in a 50-μl volume for 2 min. The reaction was stopped by the addition of 5 μl of β-mercaptoethanol. DNA

modified in vitro at G residues was prepared by the addition of 5 μl of 1% DMS (in ethanol) to ~20 μg of genomic DNA in 220 μl 20 mM Tris–HCl/5 mM EDTA (pH 8) for 20 min at 37°C. The reaction was stopped by the addition of 50 μl of buffer containing 1.5 M sodium acetate (pH 7.0) and 1 M β-mercaptoethanol. For G/A and C/T sequence ladders, 20 μg genomic DNA in 20 μl was incubated with 25 μl of formic acid (G/A) or 30 μl hydrazine (C/T) at room temperature for 4 and 6 min, respectively. Reactions were terminated by the addition of 750 μl of a solution containing 0.3 M sodium actetate (pH 7.0), 0.1 mM EDTA, and 25 μl/ml yeast tRNA. DNA was precipitated by the addition of 750 μl of ethanol. For subsequent cleavage at the modified base residues, DNA was resuspended in 400 μl of H_20, and 400 μl of 20% piperidine was added. After incubation at 94°C for 30 min, the tubes were frozen in dry ice. To remove piperidine, the solution was vacuum dried. The DNA was precipitated with ethanol and subjected to the linker/ligation mediated PCR method as described (Mueller and Wold 1989), with the modifications noted below. Primers used to footprint the template strand within the 5′ region of c-*myc* exon 1 were (1) 5′-TTCCCAAAGCAGAGGGCGTG-3′ (position −259 to −240 relative to the P2 initiation site), (2) 5′-AGATCCTCTCTCGCTAATCTCCGC-3′ (position −225 to −202), and (3) 5′-CCTCTCTCGCTAATCTCCGCCCACCGGC-3′ (position −221 to −194). The hybridization temperature for primer 1 was 50°C, 60°C for primer 2, and 66°C for primer 3. For this primer set, the concentrations of dNTP and Mg^2 ions during the *Taq* polymerase amplification reaction were at 0.4 and 10 mM, respectively. Primers used to footprint $KMnO_4$ modifications of the coding strand (FP1–FP3) and noncoding strand (GP1–GP3) within the exon 1/intron 1 boundary region of the c-*myc* gene were FP1, 5′-GGTAGTCTTAAAAACCATTCCCG-3′ (position +612 to +634 relative to the P2 initiation site); FP2, 5′-CCCGTTTTCCCTCTGCCTTCTCCTCT-3′ (position +590 to +615); FP3, 5′-CCCTCTGCCTTCTCCTCTCCCATCTTGAC-3′ (position +579 to + 607); GP1, 5′-CACGAAACTTTGCCCATAGC-3′ (position +191 to +210); GP2, 5′-GGGCACTTTGCACTGGAACTTACAAC-3′ (position +218 to +243); and GP3, 5′-TGCACTGGAACTTACAACACCCGAGCAAGG-3′ (position +226 to +255). The hybridization temperature was 55°C for FP1, 64°C for FP2, 68°C for FP3, 55°C for GP1, 61°C for GP2 and 65°C for GP3. Only primers used in the final labeling reaction were gel purified. For all footprinting studies, the Sequenase reaction was performed at 37°C.

Nuclear run-on transcription assays

Isolation of nuclei from cells lines and nuclear run-on assays were performed as described (Bentley and Groudine 1986), with the following modifications: After the run-on reaction and DNase I treatment of nuclei, RNA was isolated by the RNAzol method: 900 μl RNAzol (Cinna/Biotecx) and 90 μl of chloroform were added to the run-on reaction and vortexed vigorously. The nucleic acids were subsequently recovered by precipitation with one volume of isopropanol. Unincorporated nucleotides were removed by use of a Sephadex G50 spin column or two additional ethanol precipitations. After hybridization to filter-bound, single-stranded DNA probes, GeneScreen filters were treated at 30°C for 30 min with 10 μg/ml of RNase A in 2× SSC (0.3 M NaCl, 0.03 M sodium acetate) and then washed twice in 0.1× SSC/1% SDS at 65° C. Filters were exposed to storage phosphor screens and scanned and quantified with a PhosphorImager system (Molecular Dynamics).

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Note added in proof

After this manuscript was in press, L.J. Strobl and D. Eick published nuclear run-on experiments that confirm the observation that a high density of polymerase II in the promoter–proximal region accounts for the excess of c-*myc* exon 1 transcription in HL-60 cells (Strobl and Eick 1992). Hold-back of RNA polymerase II at the transcription start site mediates down-regulation of c-*myc* in vivo. (*EMBO J.* **11:** 3307–3314).

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