MOLECULAR AND CELLULAR BIOLOGY, Feb. 1988, p. 867–874
0270-7306/88/020867-08$02.00/0

Vol. 8, No. 2

Two Distinct Mechanisms of Transcriptional Control Operate on c-*myc* during Differentiation of HL60 Cells

ULRICH SIEBENLIST,[1]* PETER BRESSLER,[1] AND KATHLEEN KELLY[2]

Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases,[1] and Immunology Branch, National Cancer Institute,[2] Bethesda, Maryland 20892

Received 10 September 1987/Accepted 25 November 1987

We examined the mechanisms that control the downregulation of the c-*myc* mRNA during differentiation of HL60 cells. On treatment with dimethyl sulfoxide, HL60 cells downmodulated their steady-state c-*myc* message levels, ceased to proliferate, and underwent terminal differentiation. In nuclear run-on assays in which distinct segments of the c-*myc* gene were used as probes, an increased blocking to elongation of nascent c-*myc* transcripts was shown during the early phase of differentiation. During a later phase, however, a loss of transcriptional initiation was observed. This loss of promoter activity correlated well with dramatic changes in the chromatin structure of the c-*myc* gene, as determined by DNase I-hypersensitive site analysis. In particular, two hypersensitive sites near the two major c-*myc* promoters disappeared at the time that promotion abated. The newly described, later-acting negative transcriptional control of c-*myc* also correlated temporally with the inability to reverse the downregulation of the c-*myc* message quickly on withdrawal of the differentiating agent. Therefore, a terminal step during differentiation may be linked to the later-acting mode of transcriptional regulation of c-*myc*. The evidence presented in this report has implications for tumorigenesis in Burkitt lymphomas, in which the germ line, nontranslocated c-*myc* allele is transcriptionally silent.

The promyelocytic leukemia cell line HL60 can be differentiated terminally in vitro. Treatment with agents such as dimethyl sulfoxide (DMSO) (11), N^6,O^2-dibutyryl adenosine 3′,5′-cyclic monophosphate (dbcAMP) (8), and retinoic acid (6) results in the expression of granulocytic markers in HL60 cells, while exposure to vitamin D (1, 39) or phorbol esters (45) leads to the expression of macrophage-associated markers. HL60 cells exposed to these inducers undergo growth arrest and ultimately reach a terminally differentiated state. The c-*myc* steady-state mRNA decreases to very low levels within a few hours after the addition of differentiating agents (3, 20, 22, 27, 38, 44, 48, 53, 55, 56). It has been suggested that this decrease may be a necessary step in cellular differentiation and growth arrest of many terminally differentiating cells, and recent data on MEL cell differentiation support this view. The presence of a transfected and constitutively expressed *myc* gene appears to prevent differentiation of MEL cells (13, 18, 33, 43). Thus, it is important to understand the mechanism by which differentiating cells downregulate the expression of their *myc* gene. In recent reports (3, 22, 40, 41) it has been suggested that this is accomplished in HL60 cells primarily via an elongation block of nascent RNA transcripts rather than via a repression of transcription starts. Notwithstanding, chromatin structure changes have been observed to occur upstream of the c-*myc* promoters in differentiating HL60 cells, although there exist contradictory data on the exact nature of these changes (3, 20, 22, 28, 29). Chromatin changes in presumed regulatory regions of the c-*myc* gene might be expected to affect promoter function, and analysis of Burkitt lymphoma cells provides some evidence for this. Burkitt lymphoma cells are characterized by a translocation of the c-*myc* gene into one of the immunoglobulin gene loci (36). The translocated allele is highly transcribed, but the germ line, nontranslocated allele of c-*myc* is usually not transcribed (36, 42, 52). The downregulation of the normal c-*myc* allele in Burkitt lymphoma cells is accompanied by differences in its chromatin structure as compared with that of actively transcribed and unrearranged c-*myc* alleles, suggesting that these changes are associated with promoter activity (21, 47). To understand why the normal c-*myc* allele in Burkitt lymphoma cells is transcriptionally silent, it is necessary to elucidate the mechanisms that underlie negative control. In this study we determined by nuclear run-on analyses, the changes in transcription through various segments of the c-*myc* locus, and by examining DNase I hypersensitivity we determined the changes in chromatin structure during the course of HL60 differentiation. In addition to the recently documented block in elongation (3, 22), we describe a later-acting, second, and novel regulatory mechanism which operates by downregulating the initiation of c-*myc* transcripts. This negative control appears to correlate temporally with the loss of specific DNase I-hypersensitive sites and may be linked to a terminal step during differentiation. As shown here, the later-acting mechanism of downregulation is not readily reversible, whereas the mechanism that operates initially early during differentiation is reversible.

MATERIALS AND METHODS

Cell culture. HL60 cells were kindly provided by T. R. Breitman and were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and penicillin, streptomycin, and gentamicin. HL60 cells were induced to differentiate with 1.3% DMSO or with 500 μM dbcAMP at a starting cell concentration of 2×10^5 to 3×10^5 per ml. To remove DMSO during the course of activation, cells were washed 2 to 3 times in large volumes of medium at room temperature. The Nitro Blue Tetrazolium (NBT) reduction assay was performed essentially as described previously (51). About 2×10^6 cells were incubated with NBT (final concentration,

* Corresponding author.

0.1%) and phorbol myristate acetate (200 ng) in 1 ml of phosphate-buffered saline for 15 min at 37°C. The percentage of cells that contained intracellular reduced blue-black formazan deposits was then determined on safranin (0.2%)-stained cytospin preparations of the incubated cells.

RNA analysis. Total cellular RNA was isolated by guanidine isothiocyanate extraction and cesium chloride gradient centrifugation (9), size fractionated on agarose-formaldehyde gels, and transferred to nitrocellulose (37). A *Cla*I-*Eco*RI fragment, which contains the third exon of c-*myc* (2), was used as a probe to detect the c-*myc* message; the beta-2 microglobulin probe was a 600-base-pair cDNA fragment (50). All probes were nick translated by standard techniques.

Nuclear run-on analysis. A total of 1×10^7 to 2×10^7 cells were washed twice in cold phosphate-buffered saline, suspended in 3 ml of 0.3 M NIB (0.3 M sucrose, 10 mM Tris [pH 7.4], 5 mM $MgCl_2$, 0.2% Nonidet P-40), underlayed with 2.5 ml of 0.8 M NIB (0.8 M sucrose, 10 mM Tris [pH 7.4], 5 mM $MgCl_2$), and pelleted at 1,500 × *g* for 15 min. Nuclei were washed once in storage buffer (35% [vol/vol] glycerol, 50 mM Tris [pH 7.4], 5 mM $MgCl_2$, 0.1 mM EDTA), suspended in 100 μl of storage buffer, and stored at −70°C. Run-on reactions and purification of labeled RNA were done essentially as described previously (26). A total of 5 μg of linearized, denatured plasmid DNA per slot or 2.5 μg of M13 DNA per slot was transferred to nitrocellulose by using a slot blot apparatus. Filters were prehybridized overnight in 10 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; pH 7.4]–0.2% sodium dodecyl sulfate–10 mM EDTA–300 mM NaCl. Run-on transcripts were hybridized in the same buffer at 65°C for approximately 48 h. Hybridizations typically contained 5×10^6 to 10×10^6 cpm in a total volume of 1 ml. For the analysis of HL60 cells during the course of differentiation, approximately equivalent counts were used for hybridization for each time point in a given study. Filters were washed multiple times in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 65°C, followed by incubation with 1 μg of DNase-free RNase A (Cooper Scientific) per ml in 0.2× SSC at 37°C for 30 min. Single-stranded M13 (mp11) DNA probes contained the following c-*myc* fragments in sense (probes a, c, and e) and antisense (probes b, d, and f) orientations: a *Pvu*II fragment that contained the first exon, the two major promoters, and some upstream sequences (probes a and b); a *Pvu*II fragment that extended from the end of the first exon into intron 1 (probes c and d); and a *Pvu*II fragment that covered the rest of intron 1 and that extended into exon 2 (probes e and f) (2). These three fragments were contiguous and were of approximately similar lengths. In addition, we employed several plasmid clones as probes that contained the following fragments: the *Cla*I-*Eco*RI fragment containing the third c-*myc* exon (2), an HLA cDNA clone (49), and pBR322 as a negative control. Densitometric analysis was performed on a densitometer (DU-8; Beckman Instruments, Inc., Fullerton, Calif.).

DNase I-hypersensitive site analysis. The isolation of nuclei, digestion with DNase I, and the subsequent purification and analysis of the DNA have been described in detail previously (47). Briefly, nuclei were isolated from cells by detergent lysis with Nonidet P-40, purified by sucrose gradient centrifugation, suspended at a concentration of between 2×10^7 and 5×10^7 nuclei per ml, and then digested with DNase I for 3 min at room temperature. The amounts of DNase I used are indicated in the figure legends. A *Pvu*II-*Xba*I fragment that extended from the end of exon 1 into the first intron of c-*myc* was used as a probe for most analyses, and a *Cla*I-*Eco*RI fragment that contained the third c-*myc* exon was also used (2).

RESULTS

Transcriptional regulation of the c-*myc* mRNA during the course of differentiation of HL60 cells. The promyelocytic leukemia cell line HL60 was treated with DMSO to initiate terminal differentiation. Between 48 and 72 h after incubation with 1.3% DMSO, HL60 cells cease to proliferate (11, 23–25, 57, 58) and begin to reduce NBT, which is a measure of granulocytic cell function (5, 54). Whereas only about 2% of the untreated HL60 cells reduced NBT, approximately 57 and 72% of the cells could do so after 72 and 96 h of differentiation, respectively. The c-*myc* steady-state mRNA levels were analyzed during the course of differentiation (Fig. 1). As reported previously (20, 22, 29, 56), c-*myc* expression is dramatically reduced within a few hours of exposure to DMSO and remains extremely low thereafter. Downregulation of c-*myc* to barely detectable levels occurs in spite of the amplified c-*myc* gene in HL60 cells, which results in very high levels of constitutively expressed c-*myc* mRNA in uninduced cells (10, 14). Some low level of message remained during the whole course of differentiation, possibly because not all cells differentiate (24, 51) (Fig. 1A). Also, there was some restimulation of c-*myc* expression after about 8 h of differentiation, which is reminiscent of the biphasic behavior of c-*myc* expression during differentiation of MEL cells (34) or of dbcAMP-treated HL60 cells (38). On the other hand, restimulation of the c-*myc* message in MEL cells is much more pronounced and may be unrelated mechanistically. Posttranscriptional regulation of the c-*myc* gene has been demonstrated in several systems (16, 17, 19, 32, 40), and cytoplasmic degradation of c-*myc* messages may be prevented by protein synthesis inhibitors like cycloheximide (15). To test whether c-*myc* mRNA molecules could be rescued from such potential cytoplasmic degradation, we

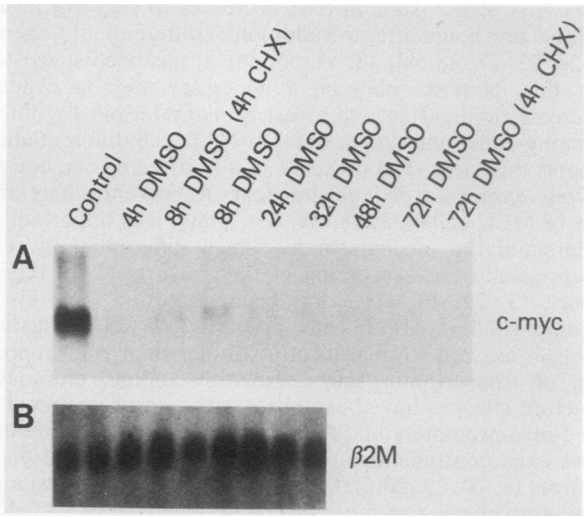

FIG. 1. Northern blot analysis of the c-*myc* message during the course of differentiation of HL60 cells. (A) Total cellular RNA was extracted from differentiating HL60 cells at the indicated times after the addition of DMSO and subsequently probed for c-*myc* mRNA. Also, cycloheximide (CHX) was added at 10 μg/ml for the final 4 h to cells that were exposed to DMSO for a total of 8 or 72 h. The control lane represents undifferentiated cells. (B) Hybridization of RNAs shown in panel A to beta-2 microglobulin (β2M), to ensure that approximately equal amounts of RNA were loaded in each lane.

added cycloheximide for the final 4 h to HL60 cells that were treated with DMSO for 8 or 72 h (Fig. 1A). This treatment did not restore uninduced levels of c-*myc*, suggesting that the downregulation of c-*myc* is unlikely to be purely a consequence of increased cytoplasmic degradation of the message.

To understand the mechanisms that regulate the c-*myc* steady-state mRNA levels during differentiation of HL60 cells, we performed nuclear run-on experiments in which nascent RNA chains were extended in isolated nuclei to measure transcription rates (26). It has been reported recently (3, 22, 40, 41) that the transcription rates of the first exon of c-*myc* are higher than are those for the rest of gene, and this has been attributed to a putative transcriptional elongation block near the boundary of the first exon and intron. Moreover, this elongation block is much more pronounced when HL60 cells are differentiated, thus leading to the downregulation of the full-length c-*myc* message (3, 22). Therefore, we probed the nuclear run-on transcripts with several probes derived from different regions of the c-*myc* gene. The location of these probes on a map of c-*myc* is shown in Fig. 2A. Probes b, d, and f were single-stranded probes which hybridized to the sense transcripts; and probes a, c, and e hybridized to antisense transcripts. Probe p was

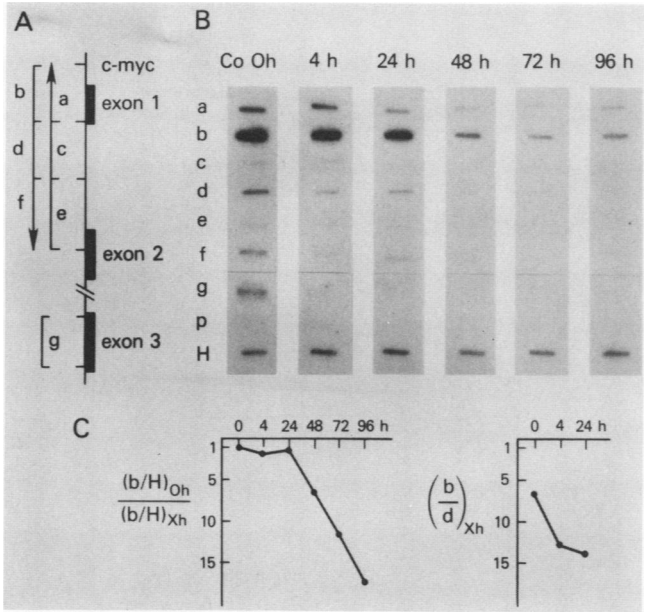

FIG. 2. Nuclear run-on analysis of c-*myc* during the course of HL60 cell differentiation. Nuclear run-on transcripts were probed with distinct segments of the c-*myc* gene (see text). (A) The probes are shown relative to a map of the c-*myc* locus. The single-stranded phage clones containing probes a, c, and e hybridized to antisense transcripts, whereas clones containing probes b, d, and f hybridized to sense transcripts. Probe g was a plasmid clone which covered the third exon. (B) Hybridization of the nuclear run-on transcripts to probes which were slot blotted. In addition to the probes shown in panel A, we also used a plasmid probe (p) as a negative control and an HLA cDNA plasmid clone (H) as a normalization control. The times of exposure to DMSO are shown at the top of the gels. (C) Results of a densitometric analysis of panel B. The left graph shows the decline of transcription through exon 1 with differentiation, which was normalized to the HLA signal: b/H at 0 h of differentiation divided by b/H at the indicated times. The graph emphasizes ratios rather than amounts. The right graph shows the decreasing elongation efficiency of nascent transcripts by comparing hybridization signals of probe b divided by probe d at the indicated times.

plasmid DNA and thus represents a negative control, and probe H was an HLA cDNA segment that was the positive control. Differentiation for 4 h led to a relatively small decrease of transcriptional activity in the first exon (two- to threefold in three separate experiments), but essentially no transcriptional activity was seen within the third exon (probe g; Fig. 2B). At approximately 24 h of differentiation, the transcription rate through the first exon increased to predifferentiation levels. This increase, following the decrease seen at 4 h, may be responsible for the small, transient increase in the steady-state message seen 8 h after DMSO treatment (Fig. 1). Consistent with results of previous reports (3, 22), little transcriptional activity was detectable in the first intron and sequences further 3′, suggesting that differentiation leads to an enhancement of the proposed transcriptional block somewhere near the first exon and intron border. Although hybridization with probes downstream of the elongation block was very weak at or after 4 h of differentiation, the data in Fig. 2B indicate that there was a further decrease of transcriptional activity 3′ of the first intron and exon border (compare strengths of signals to probes d and g at 0 h of differentiation with those at 4 and 24 h). We observed some antisense transcription complementary to probe a, which contained the first exon, in agreement with results of a previous report (3). Continued differentiation for a minimum of 48 h, however, led to a dramatic loss of transcriptional activity even within the first exon, suggesting the downregulation of transcriptional initiation. This result has not been observed previously, possibly because analyses were done at times such that the differentiation process had not yet sufficiently progressed (3, 22). The use of retinoic acid (3) as a differentiating agent or the nature of the HL60 subline are also potential sources for the differences. Although other explanations cannot be excluded, it is possible that the aforementioned sources of differences slightly delayed the exact time at which the later changes take place, necessitating longer differentiation studies than have been performed in a previous study (3).

These data reveal two apparently distinct transcriptional mechanisms for the downregulation of c-*myc*: an early increased pausing or blocking of transcriptional elongation and a later loss of transcriptional initiation. The increased blocking of elongation may well be maintained continuously throughout the while time course of differentiation, but the nuclear run-on signals obtained after 48 h were too weak to accurately assess relative transcription rates in different gene segments. The weaker but still detectable transcriptional activity of exon 1 even at 96 h of differentiation was likely due, at least in part, to some cells which did not properly differentiate (24, 51).

Results obtained from densitometric analyses of data presented in Fig. 2B are given in Fig. 2C. The graph on the left in Fig. 2C shows the loss of transcriptional activity within the first exon by comparing the hybridization signal of normalized probe b at 0 h of differentiation with that at various points during the time course of differentiation. We observed a rapid and progressive loss of transcriptional activity through the first exon by about 48 h of differentiation, and by 96 h the rate decreased at least 15-fold. In three separate experiments a similar decline was shown; after 96 h a minimal 10-fold decrease was observed. HLA transcription is a valid control index since HLA transcript levels do not change during the time course of differentiation, as judged by Northern blot analyses, relative to the total amount of RNA that was loaded and also relative to the level of beta-2 microglobulin transcripts (data not shown). The graph on the

right in Fig. 2C is a measure of the transcriptional block around the end of exon 1, which was made by comparing the hybridization signal to probe d with that to probe b at the indicated times during differentiation. During later stages of differentiation the signals were too weak to allow quantitation. Since the exact borders of the elongation block are not known, this analysis only yielded an estimate of the increase in the block. Nonetheless, we saw an approximately 6-fold greater transcriptional activity for probe b as compared with that for probe d in undifferentiated cells, which increased to about 14-fold after 24 h or DMSO treatment. These quantitations are in reasonably good agreement with those given in a previous report (3).

Nuclear run-on analyses with additional, different probes revealed only a small amount of sense transcription upstream of exon 1 (data not shown), suggesting a relatively weak promotion in our cells from the P0 promoter (3, 22, 40) which lies approximately 600 base pairs upstream of P1 and P2 (4). Therefore the transcriptional activity detected in exon 1 initiated almost entirely from the P1 and P2 promoters, and in particular from P2, which is stronger (3, 7). This further implies that the substantial decrease in transcription through exon 1 that we observed must necessarily be due primarily to a loss of transcription from promoter P2.

Chromatin structural analysis of the c-*myc* locus during HL60 differentiation. To identify DNA sequences that may be associated with transcriptional regulation of the c-*myc* gene, we performed DNase I-hypersensitive site analyses. As reported previously by us (47), c-*myc* chromatin displays a number of DNase I-hypersensitive sites which occur at or near sequences that are likely to be involved with transcriptional regulation. The perturbations in the chromatin which give rise to such DNase I-hypersensitive regions are possibly the result of nonhistone proteins that are bound to the DNA. We have identified several hypersensitive sites in the 5′ region of the gene, which is the putative regulatory segment of c-*myc*. These sites occur in all cells which we have analyzed (46, 47). In previous reports (3, 20, 22, 28, 29), only partial and even somewhat conflicting information on the chromatin structure of c-*myc* in differentiating HL60 cells has been provided. In the present study we systematically analyzed the chromatin structure in detail during the course of differentiation. The undifferentiated HL60 cells exhibited a pattern of hypersensitive sites that was essentially the same as that described for other cells (21, 46, 47), except that site III_3 was much stronger in HL60 cells (Fig. 3A and B). This site was weak and thus was unlabeled in our prior report (47). Differentiation of HL60 cells for 50 h, however, caused dramatic changes in the chromatin structure. Hypersensitive sites II_2, III_1, and III_2 were much reduced or disappeared (as indicated by the arrows in Fig. 3A), whereas sites I and III_3 remained and, as such, represented internal controls. To detect the change in hypersensitive site III_2, a high-resolution electrophoretic analysis was required to distinguish III_2 from the unchanged site III_3. Also, since site III_1 was relatively weak even before differentiation, clear detection of its change required strong signals on the autoradiograph.

The changes described above occurred not only with DMSO but also with the effective differentiating agent dbc AMP (8, 38, 53), confirming the observation. We noted new, weak hypersensitive sites that were especially evident in dbcAMP-treated cells, which appeared or were uncovered near but not identical with sites II_2 and III_1.

As discussed above, c-*myc* transcripts disappeared early, while initiation of transcription, as determined by transcription through exon 1, declined only after about 48 h of differentiation. To test whether the observed changes in the chromatin structure could be correlated with this change in transcription initiation, we assayed the chromatin structure at 24 and 72 h of differentiation (Fig. 4). After 24 h only site II_2 was reduced considerably, but little else changed, except that site III_1 appeared to have decreased somewhat. No changes were observed after 4 h of differentiation (data not shown). The chromatin structure seen after 72 h resembled that seen after 50 h of differentiation, except that site III_3 appeared to have diminished as well. This suggests that the disappearance of sites III_1 and III_2, in particular, is well correlated temporally with the loss in transcription initiation at about 48 h of differentiation. These two regions are located just upstream of the two major c-myc promoters P1 and P2, respectively. The decrease of intensity in site II_2 preceded slightly the change in transcriptional activity in exon 1. As reported previously (3), site II_2 may be associated with the weaker P0 promoter, and thus may be related to its activity. Because site II_2 was a well-resolved gel band and decreased dramatically somewhat earlier during differentiation, this may be the reason that only this change has been noted consistently (3, 20, 22, 29).

Site I and several weaker sites downstream of the promoters did not change appreciably (data not shown), although

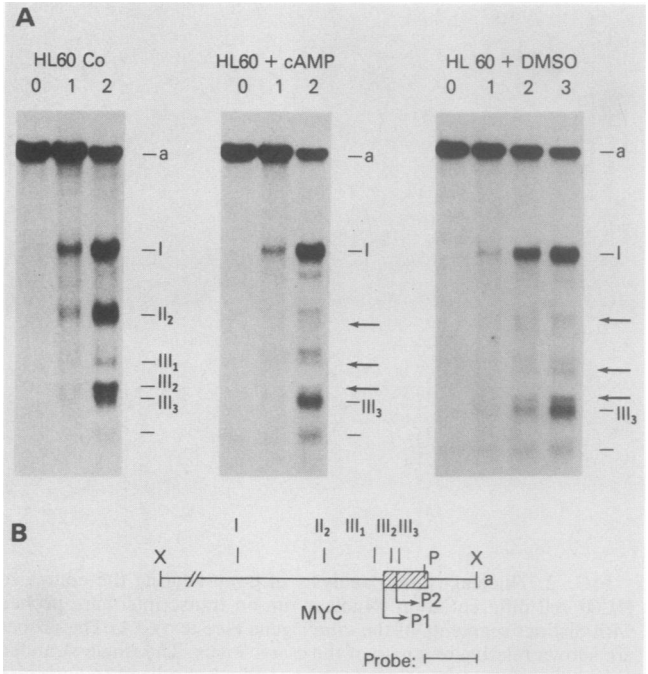

FIG. 3. DNase I-hypersensitive site analysis of c-*myc* before and after a 2-day differentiation of HL60 cells with DMSO or dbcAMP. (A) HL60 nuclei were digested with increasing amounts of DNase I (see text). Lanes 0, No DNase I; lanes 1, 3.74 U DNase I per ml; lanes 2, 14.96 U of DNase I per ml; lanes 3, 29.92 U of DNase I per ml. Control (Co) cells were undifferentiated. The hypersensitive sites are marked with roman numerals I, II, and III. An additional weak hypersensitive site is marked by a line below site III_3. Arrows indicate those sites that were markedly diminished in cells that were differentiated for 50 h relative to control cells. a represents the genomic *Xba*I (X) fragment which hybridized to the probe shown in panel B. (B) Locations of the hypersensitive sites relative to a map of the 5′ part of the c-*myc* locus. P1 and P2 refer to the two c-*myc* promoters, and the shaded, boxed area represents exon 1. P, *Pvu*II.

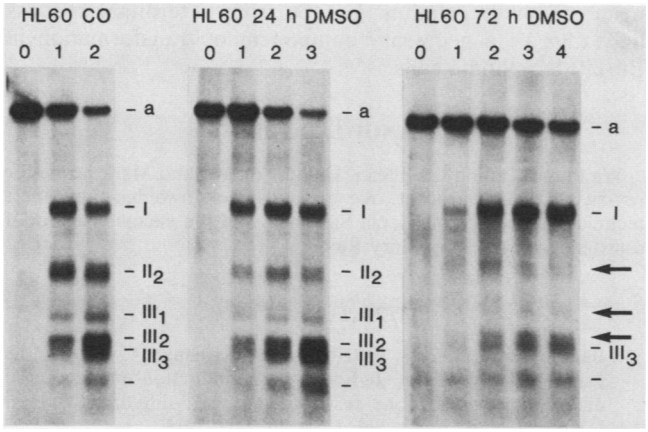

FIG. 4. DNase I-hypersensitive site analysis of c-*myc* in undifferentiated cells and in HL60 cells that were differentiated for 24 and 72 h. Nuclei from undifferentiated (control [CO]) HL60 cells or from cells that were differentiated for 24 or 72 h with DMSO were digested with increasing amounts of DNase I (see text). The DNase I concentrations are as given in the legend to Fig. 3; in addition, for lane 4 we used 33.66 U of DNase I per ml of nuclei. DNA was digested with *Xba*I and probed as described in the legend to Fig. 3.

differentiation with retinoic acid has been reported (3) to increase site V, which is located within the first intron. The reasons for this difference are unclear, but they may be related to the differentiation agent.

Loss of reversibility to high c-*myc* mRNA levels correlates with chromatin changes. The dramatic chromatin changes observed after 48 h of differentiation suggest that c-*myc* entered a state which was not easily reversible in terms of transcriptional activity. To test this hypothesis, we attempted a reversal of the differentiation course by withdrawing the differentiating agent DMSO, which was accomplished by repeatedly washing the cells. We compared the levels of steady-state c-*myc* mRNA after 24 and 72 h of differentiation with the levels obtained after an additional 4 h of differentiation in the absence of DMSO. The 24- and 72-h time points were well before and after the stage at which the changes in chromatin structure and the decrease of exon 1 transcription were easily demonstrated. A remarkably quick return to predifferentiation levels (Fig. 5, control lane) of c-*myc* expression if DMSO was washed out after 24 h of differentiation is shown, but very little c-*myc* expression could be restimulated during 4 h after a differentiation for 72 h. The low level of c-*myc* mRNA which reappeared at this later stage was likely due to the fact that some cells were not properly differentiated (24, 51). Results of this experiment suggest that the change in chromatin structure, the decline in transcriptional initiation, and the lost ability to reverse the downregulation of c-*myc* message rapidly are all correlated. This mode of regulation, which could be demonstrated as early as after 48 h of differentiation, may constitute a necessary step in the commitment to differentiation and growth arrest.

DISCUSSION

In this report we have shown that two distinguishable modes of regulation operate on c-*myc* during differentiation of HL60 cells with DMSO. During the early stages of differentiation, transcriptional elongation through the c-*myc* gene appeared to encounter increased blocking near the end of the first exon, thus preventing the formation of full-length c-*myc* transcripts (3, 22). After about 48 h of differentiation, however, another control mechanism occurred in which initiation of c-*myc* transcription was progressively downregulated, as judged by the decrease of nuclear run-on transcription through the first exon. This later-acting mode of negative control coincided with dramatic changes in chromatin structure. In particular, two hypersensitive sites near the c-*myc* promoters (III_1 and III_2) decreased at about the time that a loss of transcription initiation was observed. This newly described downregulation also correlated well with the inability of differentiated HL60 cells to upregulate c-*myc* quickly after DMSO was withdrawn. Prior to this step, c-*myc* was easily reexpressed in the absence of DMSO. Since irreversibility of the differentiation process occurs at about the same time (8, 24, 25, 51, 58), these data suggest that there is a connection between the loss of c-*myc* promoter function and the commitment to terminal differentiation and growth arrest.

The temporal correlation in the differentiation of HL60 cells between the decline of hypersensitive sites III_1 and III_2 and the decreased transcription rate through exon 1 is suggestive of a loss of promoter function due to changes in *trans*-acting complexes. Additional evidence links the promoter-associated sites III_1 and III_2 with the potential activity of the P1 and P2 promoters, respectively. The relatively increased intensity of site III_1, as compared with that of site III_2, in a Burkitt lymphoma is accompanied by increased transcription from the P1 promoter compared with that from the P2 promoter (47, 52). Furthermore, the relative intensities of these two sites appears to correlate well with the relative activities of the associated promoters (3, 7, 47, 52). Thus, the decline of sites III_1 and III_2 during differentiation in HL60 cells coincides with and is consistent with the loss of P1 and P2 promoter function. Although the stronger P2 promoter is primarily responsible for the transcriptional activity through exon 1 (3, 7), the chromatin data may suggest that both promoters lose their initiation capability.

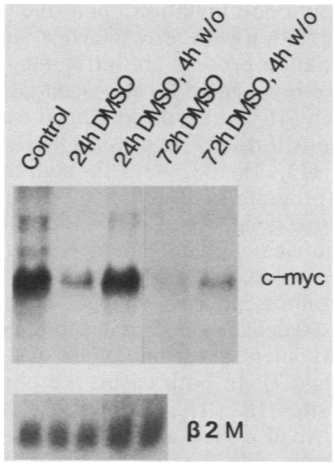

FIG. 5. The downregulation of c-*myc* mRNA levels in HL60 cells can be easily reversed after 1 day but not after 3 days of differentiation with DMSO. The steady-state c-*myc* mRNA levels in HL60 cells that were differentiated for 24 or 72 h with DMSO were compared with those in which DMSO was present for 24 or 72 h, but removed for the next 4 h (24h DMSO, 4h w/o, and 72h DMSO, 4h w/o, respectively). Control refers to undifferentiated cells. Hybridization of the same RNAs to beta-2 microglobulin (β2M) showed that approximately equal amounts of RNA were loaded in each lane.

Hypersensitive site II_2 showed a dramatic decline in strength following DMSO treatment, which was somewhat before we detected a decline in exon 1 transcription. Site II_2 may be related to the function of the weaker P0 promoter (3), which is located upstream of promoters P1 and P2, near site II_2 (4). This does not rule out a role for II_2 in regulating the promoter activity of P1 and P2.

The nuclear run-on data revealed a relatively small, two- to threefold, change in transcriptional initiation at 4 h of differentiation, although by 24 h the level of initiation increased back to the level found in undifferentiated cells. Since there were no noticeable changes in chromatin structure at 4 h, it appears that the promoter activity of the c-*myc* gene can be regulated, at least to some degree, in ways which are not easily detected by studies of hypersensitive DNase I hypersensitivity. It is of interest that quiescent normal T cells show little transcription of exon 1 but, nevertheless, exhibit all hypersensitive sites that were seen in actively transcribing cells (K. Kelly and U. Siebenlist, unpublished data). Thus, the presence of hypersensitive sites near the c-*myc* promoters appears to be necessary but not sufficient for strong transcriptional activity.

The inability to quickly reverse the c-*myc* mRNA levels by withdrawing DMSO after 72 h of differentiation and the ability to do so after only 24 h of exposure correlates well with the chromatin changes and the cessation of promoter activity that was detectable at or after about 48 h of differentiation. It thus appears likely that such changes are responsible for preventing a restimulation of c-*myc* expression at later times. Interestingly, the period from 48 to 72 h constitutes what has been termed the commitment phase of differentiation. That is, a large number of cells progress to a stable differentiated phenotype after this phase, even in the absence of any differentiating agents (8, 12, 24, 25, 51, 58). This was not the case after 24 h of differentiation. In our experiments we did not address the question of whether the changes in c-*myc* that were observable after 48 h of differentiation are directly related to terminal growth arrest and induction of a stable differentiated phenotype. On the other hand, the temporal correlation of the late changes in chromatin, transcriptional initiation, and the reversibility of c-*myc* downregulation with growth arrest and irreversibility of the differentiation process are intriguing. The downregulation of the c-*myc* gene is an essential component of differentiation in MEL cells, as continued expression from a transfected, constitutively expressed *myc* gene prevents differentiation (13, 18, 33, 43). In addition, the normal differentiation program of B cells is perturbed by the presence of the c-*myc* gene, when it is under the control of the immunoglobulin heavy-chain enhancer in transgenic mice (35).

The differentiation-induced, changed chromatin structure of c-*myc* in HL60 cells is very similar to the one seen on the transcriptionally silent germ line allele of c-*myc* in Burkitt lymphoma cells (47). In both cases, we observed a loss in intensities of sites II_2, III_1, and III_2 relative to those in actively transcribed c-*myc* alleles. This may indicate that Burkitt lymphoma cells or their precursors undergo a process similar to terminal differentiation, which is associated with the loss of expression from the germ line c-*myc* allele (30, 31). It is possible that such a process was meant to eliminate these cells; but instead, these cells may have survived and expanded, at least in part, because of continued expression of c-*myc* from the translocated allele. This further implies that the translocation event deregulated the translocating c-*myc* gene by interfering with some mechanism of downregulation. Escape from a terminal process may thus be a necessary component of transformation in Burkitt lymphoma cells.

ACKNOWLEDGMENTS

We thank Anthony S. Fauci, David Levens, and Mark Minie for reviewing the manuscript. We gratefully acknowledge the expert technical assistance of Nicola Salvatore and the excellent editorial assistance provided by Mary Rust.

LITERATURE CITED

1. **Bar-Shavit, Z., S. L. Teitelbaum, P. Reitsma, A. Hall, L. E. Pegg, J. Trial, and A. J. Kahn.** 1983. Induction of monocytic differentiation and bone resorption by 1,25-dihydroxyvitamin D3. Proc. Natl. Acad. Sci. USA **80**:5907–5911.
2. **Battey, J., C. Moulding, R. Taub, W. Murphy, T. Stewart, H. Potter, G. Lenoir, and P. Leder.** 1983. The human c-myc oncogene: structural consequences of translocation into the IgH locus in Burkitt lymphoma. Cell **34**:779–787.
3. **Bentley, D. L., and M. Groudine.** 1986. A block to elongation is largely responsible for decreased transcription of c-myc in differentiated HL60 cells. Nature (London) **321**:702–706.
4. **Bentley, D. L., and M. Groudine.** 1986. Novel promoter upstream of the human c-*myc* gene and regulation of c-*myc* expression in B-cell lymphomas. Mol. Cell. Biol. **6**:3481–3489.
5. **Breitman, T. R., B. R. Keene, and H. Hemmi.** 1983. Retinoic acid-induced differentiation of fresh human leukaemic cells and the human myelomonocytic leukemia cell lines, HL-60, U-937, and THP-1. Cancer Surv. **2**:263–291.
6. **Breitman, T. R., S. E. Selonick, and S. J. Collins.** 1980. Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. Proc. Natl. Acad. Sci. USA **77**:2936–2940.
7. **Broome, H. E., J. C. Reed, E. P. Godillot, and R. G. Hoover.** 1987. Differential promoter utilization by the c-*myc* gene in mitogen- and interleukin-2-stimulated human lymphocytes. Mol. Cell. Biol. **7**:2988–2993.
8. **Chaplinski, T. J., and J. E. Niedel.** 1982. Cyclic nucleotide-induced maturation of human promyelocytic leukemia cells. J. Clin. Invest. **70**:953–964.
9. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
10. **Collins, S., and M. Groudine.** 1982. Amplification of endogenous myc-related DNA sequences in a human myeloid leukaemic cell line. Nature (London) **298**:679–681.
11. **Collins, S. J., F. W. Ruscetti, R. E. Gallagher, and R. C. Gallo.** 1978. Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. Proc. Natl. Acad. Sci. USA **75**:2458–2462.
12. **Cooper, R. A., S. H. Ip, P. A. Cassileth, and A. L. Kuo.** 1981. Inhibition of sterol and phospholipid synthesis in HL-60 promyelocytic leukemia cells by inducers of myeloid differentiation. Cancer Res. **41**:1847–1852.
13. **Coppola, J. A., and M. D. Cole.** 1986. Constitutive c-myc oncogene expression blocks mouse erythroleukemia cell differentiation but not commitment. Nature (London) **320**:760–763.
14. **Dalla-Favera, R., F. Wong-Staal, and R. C. Gallo.** 1982. Onc gene amplification in promyelocytic leukaemia cell line HL-60 and primary leukaemic cells of the same patients. Nature (London) **299**:61–63.
15. **Dani, C., J. M. Blanchard, M. Piechaczyk, S. El Sabouty, L. Marty, and P. Jeanteur.** 1984. Extreme instability of myc mRNA in normal and transformed human cells. Proc. Natl. Acad. Sci. USA **81**:7046–7050.
16. **Dani, C., N. Mechti, M. Piechaczyk, B. Lebleu, P. Jeanteur, and J. M. Blanchard.** 1985. Increased rate of degradation of c-myc mRNA in interferon-treated Daudi cells. Proc. Natl. Acad. Sci. USA **82**:4896–4899.

17. **Dean, M., R. A. Levine, and J. Campisi.** 1986. c-*myc* regulation during retinoic acid-induced differentiation of F9 cells is post-transcriptional and associated with growth arrest. Mol. Cell. Biol. **6**:518–524.
18. **Dmitrovsky, E., W. M. Kuehl, G. F. Hollis, I. R. Kirsch, T. P. Bender, and S. Segal.** 1986. Expression of a transfected human c-myc oncogene inhibits differentiation of a mouse erythroleukemia cell line. Nature (London) **322**:748–750.
19. **Dony, C., M. Kessel, and P. Gruss.** 1985. Post-transcriptional control of myc and p53 expression during differentiation of the embryonal carcinoma cell line F9. Nature (London) **317**:636–639.
20. **Dyson, P. J., T. D. Littlewood, A. Forster, and T. H. Rabbitts.** 1985. Chromatin structure and transcriptionally active and inactive human c-myc alleles. EMBO J. **4**:2885–2891.
21. **Dyson, P. J., and T. H. Rabbitts.** 1985. Chromatin structure around the c-myc gene in Burkitt lymphomas with upstream and downstream translocation points. Proc. Natl. Acad. Sci. USA **82**:1984–1988.
22. **Eick, D., and G. W. Bornkamm.** 1986. Transcriptional arrest within the first exon is a fast control mechanism in c-myc gene expression. Nucleic Acids Res. **14**:8331–8346.
23. **Einat, M., D. Resnitzky, and A. Kimchi.** 1985. Close link between reduction of c-myc expression by interferon and G0/G1 arrest. Nature (London) **313**:597–600.
24. **Fibach, E., T. Peled, and E. A. Rachmilewitz.** 1982. Self-renewal and commitment to differentiation of human leukemic promyelocytic cells (HL-60). J. Cell. Physiol. **113**:152–158.
25. **Fibach, E., A. Treves, T. Peled, and E. A. Rachmilewitz.** 1982. Changes in cell kinetics associated with differentiation of a human promyelocytic cell line (HL60). Cell Tissue Kinet. **15**:423–429.
26. **Greenberg, M. E., and E. B. Ziff.** 1984. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. Nature (London) **311**:433–438.
27. **Grosso, L. E., and H. C. Pitot.** 1985. Transcriptional regulation of c-myc during chemically induced differentiation of HL-60 cultures. Cancer Res. **45**:847–850.
28. **Grosso, L. E., and H. C. Pitot.** 1985. Chromatin structure of the c-myc gene in HL-60 cells during alterations of transcriptional activity. Cancer Res. **45**:5035–5041.
29. **High, K. A., C. A. Stolle, J. W. Schneider, W. Hu, and E. J. Benz, Jr.** 1987. c-myc gene inactivation during induced maturation of HL-60 cells. Transcriptional repression and loss of a specific DNAse I hypersensitive site. J. Clin. Invest. **79**:93–99.
30. **Kelly, K., and U. Siebenlist.** 1985. The role of c-myc in the proliferation of normal and neoplastic cells. J. Clin. Immunol. **5**:65–77.
31. **Kelly, K., and U. Siebenlist.** 1986. The regulation and expression of c-myc in normal and malignant cells. Annu. Rev. Immunol. **4**: 317–338.
32. **Knight, E., Jr., E. D. Anton, D. Fahey, B. K. Freidland, and G. J. Jonak.** 1985. Interferon regulates c-myc gene expression in Daudi cells at the post-transcriptional level. Proc. Natl. Acad. Sci. USA **82**:1151–1154.
33. **Lachman, H. M., G. H. Cheng, and A. I. Skoultchi.** 1986. Transfection of mouse erythroleukemia cells with myc sequences changes the rate of induced commitment to differentiate. Proc. Natl. Acad. Sci. USA **83**:6480–6484.
34. **Lachman, H. M., and A. I. Sckoultchi.** 1984. Expression of c-myc changes during differentiation of mouse erythroleukemia cells. Nature **310**:592–594.
35. **Langdon, W. Y., A. W. Harris, S. Cory, and J. M. Adams.** 1986. The c-myc oncogene perturbs B lymphocyte development in E-mu-myc transgenic mice. Cell **47**:11–18.
36. **Leder, P., J. Battey, G. Lenoir, C. Moulding, W. Murphy, H. Potter, T. Stewart, and R. Taub.** 1983. Translocations among antibody genes in human cancer. Science **222**:765–771.
37. **Lehrach, H., D. Diamond, J. M. Wozney, and H. Boedtker.** 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry **16**:4743–4751.
38. **McCachren, S. S., Jr., J. Nichols, R. E. Kaufman, and J. E. Niedel.** 1986. Dibutyryl cyclic adenosine monophosphate reduces expression c-myc during HL-60 differentiation. Blood **68**: 412–416.
39. **McCarthy, D. M., J. F. San Miguel, H. C. Freake, P. M. Green, H. Zola, D. Catovsky, and J. M. Goldman.** 1983. 1,25-Dihydroxyvitamin D3 inhibits proliferation of human promyeocytic leukaemia (HL60) cells and induces monocyte-macrophage differentiation in HL60 and normal human bone marrow cells. Leukemia Res. **7**:51–55.
40. **Mechti, N., M. Piechaczyk, J. M. Blanchard, L. Marty, A. Bonnieu, P. Jeanteur, and B. Lebleu.** 1986. Transcriptional and post-transcriptional regulation of c-myc expression during the differentiation of murine erythroleukemia Friend cells. Nucleic Acids Res. **14**:9653–9666.
41. **Nepveu, A., and K. B. Marcu.** 1986. Intragenic pausing and anti-sense transcription within the murine c-myc locus. EMBO J. **5**:2859–2865.
42. **Nishikura, K., A. ar-Rushdi, J. Erikson, R. Watt, G. Rovera, and C. M. Croce.** 1983. Differential expression of the normal and of the translocated human c-myc oncogenes in B cells. Proc. Natl. Acad. Sci. USA **80**:4822–4826.
43. **Prochownik, E. V., and J. Kukowska.** 1986. Deregulated expression of c-myc by murine erythroleukemia cells prevents differentiation. Nature (London) **322**:848–850.
44. **Reitsma, P. H., P. G. Rothberg, S. M. Astrin, J. Trial, Z. Bar-Shavit, A. Hall, S. L. Teitelbaum, and A. J. Kahn.** 1983. Regulation of myc gene expression in HL-60 leukemia cells by a vitamin D metabolite. Nature (London) **306**:492–494.
45. **Rovera, G., T. G. O'Brien, and L. Diamond.** 1979. Induction of differentiation in human promyelocytic leukemia cells by tumor promoters. Science **204**:868–870.
46. **Siebenlist, U., D. B. Durand, P. Bressler, N. J. Holbrook, C. A. Norris, M. Kamoun, J. A. Kant, and G. R. Crabtree.** 1986. Promoter region of interleukin-2 gene undergoes chromatin structure changes and confers inducibility on chloramphenicol acetyltransferase gene during activation of T cells. Mol. Cell. Biol. **6**:3042–3049.
47. **Siebenlist, U., L. Hennighausen, J. Battey, and P. Leder.** 1984. Chromatin structure and protein binding in the putative regulatory region of the c-myc gene in Burkitt lymphoma. Cell **37**:381–391.
48. **Simpson, R. U., T. Hsu, D. A. Begley, B. S. Mitchell, and B. N. Alizadeh.** 1987. Transcriptional regulation of the c-myc protooncogene by 1,25-dihydroxyvitamin D3 in HL-60 promyelocytic leukemia cells. J. Biol. Chem. **262**:4104–4108.
49. **Sood, A. K., D. Pereira, and S. M. Weissman.** 1981. Isolation and partial nucleotide sequence of a cDNA clone for human histocompatibility antigen HLA-B by use of an oligodeoxynucleotide primer. Proc. Natl. Acad. Sci. USA **78**:616–620.
50. **Suggs, S. V., R. B. Wallace, T. Hirose, E. H. Kawashima, and K. Itakura.** 1981. Use of synthetic oligonucleotides as hybridization probes: isolation of cloned cDNA sequences for human beta 2-microglobulin. Proc. Natl. Acad. Sci. USA **78**:6613–6617.
51. **Tarella, C., D. Ferrero, E. Gallo, G. L. Pagliardi, and F. W. Ruscetti.** 1982. Induction of differentiation of HL-60 cells by dimethyl sulfoxide: evidence for a stochastic model not linked to the cell division cycle. Cancer Res. **42**:445–449.
52. **Taub, R., C. Moulding, J. Battey, W. Murphy, T. Vasicek, G. M. Lenoir, and P. Leder.** 1984. Activation and somatic mutation of the translocated c-myc gene in Burkitt lymphoma cells. Cell **36**:339–348.
53. **Trepel, J. B., O. R. Colamonici, K. Kelly, G. Schwab, R. A. Watt, E. A. Sausville, E. S. Jaffe, and L. M. Neckers.** 1987. Transcriptional inactivation of c-*myc* and the transferrin receptor in dibutyril cyclic AMP-treated HL-60 cells. Mol. Cell. Biol. **7**:2644–2648.
54. **Tsiftsoglou, A. S., and S. H. Robinson.** Differentiation of leukemic cell lines: a review focusing on murine erythroleukemia and human HL-60 cells. Int. J. Cell. Cloning **3**:349–366.
55. **Watanabe, T., E. Sariban, T. Mitchell, and D. Kufe.** 1985. Human c-myc and N-ras expression during induction of HL-60 cellular differentiation. Biochem. Biophys. Res. Commun. **126**:999–1005.

56. **Westin, E. H., F. Wong-Staal, E. P. Gelmann, R. Dalla-Favera, T. S. Papas, J. A. Lautenberger, A. Eva, E. P. Reddy, S. R. Tronick, S. A. Aaronson, and R. C. Gallo.** 1982. Expression of cellular homologues of retorviral onc genes in human hematopoietic cells. Proc. Natl. Acad. Sci. USA **79**:2490–2904.
57. **Yachnin, S., D. B. Toub, and V. Mannickarottu.** 1984. Divergence in cholesterol biosynthetic rates and 3-hydroxy-3-methylglutaryl-CoA reductase activity as a consequence of granulocyte versus monocyte-macrophage differentiation in HL-60 cells. Proc. Natl. Acad. Sci. USA **81**:894–897.
58. **Yen, A.** 1985. Control of HL-60 myeloid differentiation. Evidence of uncoupled growth and differentiation control, S-phase specificity, and two-step regulation. Exp. Cell Res. **156**:198–212.