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TRANSCRIPTION INTERRUPTION MAY BE A COMMON MECHANISM OF C-MYC REGULATION DURING HL-60 DIFFERENTIATION

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Human promyelocytic leukemia cells (HL-60) differentiate along a monocytoid pathway in response to recombinant human tumor necrosis factor or recombinant human interferon gamma. Together, these agents act synergistically to induce phenotypic differentiation. Since reduced expression of mRNA for the proto-oncogene c-myc correlates with differentiation of HL-60 cells induced by other agents, we tested the abilities of tumor necrosis factor and interferon gamma to regulate expression of c-myc mRNA. Tumor necrosis factor rapidly and effectively reduced c-myc mRNA levels. In contrast, interferon gamma did not affect c-myc mRNA levels, nor did it show synergy with tumor necrosis factor in reducing c-myc. Transcription run-on studies confirmed that tumor necrosis factor caused interruption of c-myc transcription after exon 1. Phorbol diesters also caused interruption of transcription of c-myc. Thus, interruption of transcription may be a common mode of regulation of c-myc during induced differentiation of HL-60 cells.

Human promyelocytic leukemia cells (HL-60) have proven to be a useful model for investigation of myelocytic and monocytic cellular differentiation

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Abbreviations used in this paper: TNF, recombinant human tumor necrosis factor alpha; IFN, recombinant human interferon gamma; PDBu, 4-beta-phorbol-12,13-dibutyrate; PMA, 4-beta-phorbol-12-myristate,13-acetate; NBT, nitroblue tetrazolium

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in vitro (1-6). These cells differentiate along a monocytoid pathway in response to activators of protein kinase C such as phorbol diesters and sn-1,2-diacylglycerols (7,8). Recently, differentiation of HL-60 cells has been demonstrated in response to two biological cytokines currently coming into clinical use, recombinant human tumor necrosis factor alpha (TNF) and recombinant human interferon gamma (IFN) (9-13). These agents induce monocytoid differentiation of the HL-60 cells as measured by functional and immunological criteria. Although each agent alone can induce differentiation, they are synergistic in combination (11,14,15,16). Therefore, HL-60 cells are a convenient system in which to study the effects of TNF and IFN, and their interactions.

HL-60 cells are also characterized by amplification and high expression of the proto-oncogene c-myc (17,18). Differentiation induced by a variety of agents is accompanied by a reduction in expression of c-myc, suggesting that continued expression of this gene is important in the maintenance of the immature phenotype (19-25). Expression of a transfected myc gene inhibits differentiation of a murine erythroleukemia cell line, indicating that attenuation of the myc gene product, a DNA binding protein, may be necessary for differentiation to occur (26-28). Previous studies showed that TNF rapidly reduced c-myc expression, whereas IFN had little effect on c-myc in HL-60 cells (29,30). We have extended these observations by studying possible synergism between TNF and IFN in the regulation of c-myc expression and by investigating the mechanism of this regulation. Bentley and Groudine demonstrated that transcription interruption after exon 1 was a mechanism of transcription regulation of c-myc in response to retinoic acid (31). We investigated this phenomenon in c-myc regulated by TNF and IFN. All studies were performed in parallel with HL-60 cells induced to differentiate by phorbol dibutyrate (PDBu), the paradigm for inducers of monocytic differentiation of HL-60 cells. Both TNF and the phorbol diesters caused interruption of transcription in a manner similar to that seen with retinoic acid, suggesting that this may be a common mechanism of c-myc regulation during HL-60 differentiation.

Methods

Cells, reagents, and probes: HL-60 cells were obtained from American Type Culture Collection (Rockville, Md.) and maintained in RPMI-1640 media supplemented with 10% fetal bovine serum (GIBCO, Long Island, N.Y.) in a humidified 5% CO_2 atmosphere at 37°C. All reagents were from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise noted. Tumor necrosis factor was a gift from Dr. Cynthia Chua (Duke University, Durham, N.C.) and Knoll Laboratories (Whippany, N.J.), and interferon-gamma was from Genentech, Inc. The c-myc exon 1 probe was a Pst I-Pst I restriction fragment from an HL-60 genomic c-myc clone, and the exon 3 probe was a ClaI-EcoRI fragment, both gifts of J. Nichols and R.E. Kaufman,[1] (Duke University).

Analysis of Differentiation: Following treatment with inducing agents, differentiation of HL-60 cells along a monocytoid pathway was confirmed by staining cells for nonspecific esterase activity using reagents supplied as a a kit (Sigma Chemical Co.). Ability to reduce NBT was measured as described (25).

RNA Isolation and Analysis: RNA was isolated by guanidinium isothiocyanate lysis of cells and centrifugation through a CsCl cushion (32,33). Northern blots and RNA dot blots were performed and hybridized with appropriate probes as previously described (25). Random labeling of probes with [^{32}P]CTP was done with a kit from Amersham (Arlington Heights, Il).

Nuclear run-on transcription: HL-60 cells were harvested at the indicated times after treatment and washed with ice cold phosphate buffered saline (pH 7.2), and nuclei were isolated according to the method of Bitter and Roeder (34). Isolated nuclei were resuspended in 40% glycerol, 20 mM Hepes pH 7.6, 2 mM $MgCl_2$, 2 mM dithiothreitol and stored at -70°C until use. Measurement of *in vitro* elongation of nascent mRNA in isolated HL-60 nuclei was performed according to the method of Grosso and Pitot (22). Run-on transcription of nascent RNA was terminated by the addition of 40 ug of DNase I. After 15 minutes, the nuclei were deproteinized at 42°C for 1 hr with 100 ug/ml proteinase K, 0.2% SDS and 5 mM EDTA. The transcription reaction was extracted with phenol/chloroform (1:1), yeast t-RNA was added and the mixture was precipitated with ethanol. The ^{32}P-labeled nuclear RNA was further precipitated with 10% trichloroacetic acid for 30 minutes at 4°C and reprecipitated with ethanol. The plasmids containing c-*myc* or control (pBR322) sequences were denatured, neutralized and spotted onto nitrocellulose filters using a slot blot apparatus (Schleicher and Schuell). The filters were subsequently prehybridized at 42°C for a minimum of 24 hours in 6 x SSC (1 x SSC contains 0.15 M NaCl and 0.015 M sodium citrate pH 7.0), 0.5% SDS, 5 X Denhardts (0.1% ficoll, polyvinyl pyrrolidone, bovine serum albumin fraction V) and 100 ug/ml denatured salmon sperm DNA. The purified ^{32}P labeled RNA was hybridized to the immobilized plasmids according to the method of Greenberg and Ziff (35).

Results

Effects of TNF and IFN on c-myc Exon 3 Expression. HL-60 cells were treated with TNF and IFN, alone and in combination, at 100 units/ml. These concentrations of TNF or IFN were shown to effectively differentiate HL-60 cells and the combination of TNF and IFN at these concentrations was deter-

[1] Nichols, J. and Kaufman, R. Manuscript in preparation.

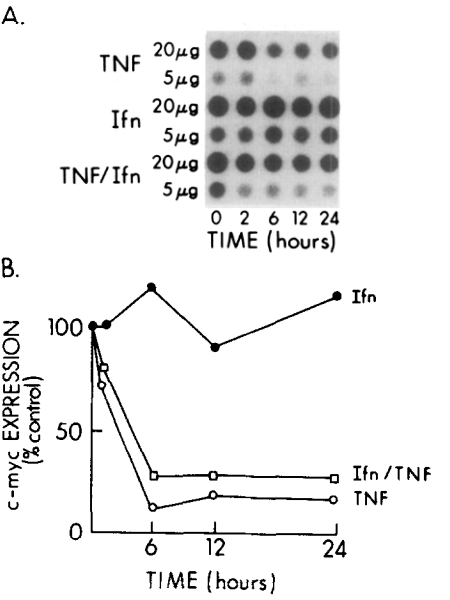

Figure 1. TNF and IFN effects on c-myc exon 3 expression. A. Representative dot blot of c-myc expression, showing agents used, amount of RNA on filter, and treatment duration. B. Average of 3 experiments demonstrating c-myc expression after treatment of HL-60 cells with indicated agents.

mined to be synergistic (16). Differentiation was confirmed by measurement of nonspecific esterase activity and ability to reduce NBT. At times from 0 to 24 hours of treatment, expression of c-myc exon 3 was measured by northern analysis or dot blot analysis of total cellular RNA. As demonstrated in figure 1, TNF caused an 80% decrease in c-myc exon 3 expression within 6 hours. In contrast, IFN caused no reduction in expression of c-myc exon 3. Although the agents act synergistically to induce phenotypic differentiation, synergy in reduction of c-myc exon 3 expression was not seen.

Differential Effect of TNF on Exon 1 and Exon 3. We next investigated the mechanism of c-myc mRNA reduction in response to TNF. Bentley and Groudine reported that retinoic acid reduced c-myc expression in HL-60 by causing transcription interruption near the exon 1-intron 1 junction (31). To determine if transcription interruption was also caused by TNF, we studied changes in mRNA transcription for both exons 1 and 3 of c-myc in differentiating HL-60 cells. Studies were also performed in parallel on cells treated with PDBu. Figure 2 shows transcription of exons 1 and 3 as a function of

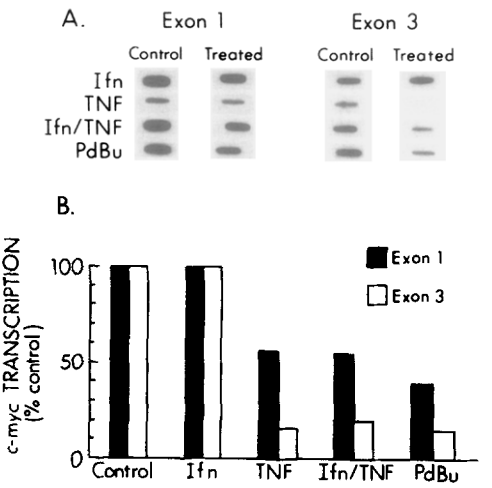

Figure 2. Transcriptional regulation of c-myc exon 1 and exon 3 by TNF and PDBu. Transcription of c-myc exons 1 and 3 was determined by elongation of nascent RNA chains in isolated nuclei. The radiolabeled RNA was hybridized to immobilized plasmids containing sequences from either exon 1 or exon 3 as indicated. No hybridization was seen to pBR322 lacking myc sequences (not shown). Nuclei were obtained from HL-60 cells treated with Ifn 100 units/ml, TNF 100 units/ml, both agents, or PDBu 100 nM for 0 or 2 hours. Transcriptional rates were determined by densitometry of autoradiographs. A. Transcription run-on study of c-myc exons 1 and 3. The differences in intensity of controls in each treatment pair is due to a difference in exposure of autoradiographs. B. Change in transcription rate of c-myc exons 1 and 3. Results are the average of 3 experiments, quantitated by densitometry of autoradiographs and expressed as percent of control (untreated cells) transcription.

time after treatment with TNF. The effects of PDBu (100 nM) are included for comparison.

Nascent RNA chains were radiolabeled by elongation in vitro and hybridized to DNA containing sequences specific for exon 1 or exon 3. As shown in figure 2, the rate of transcription of exon 1 in response to TNF was reduced by approximately 30% whereas the transcription of exon 3 was reduced by 70%, as assessed by densitometry of autoradiograms. The rate of transcription of c-myc was not altered by IFN. The combination of TNF and IFN was no more effective than TNF alone at reducing transcription of c-myc. Alpha-amanitin (2 ug/ml) eliminated all signals, confirming that RNA polymerase II specific transcription was being detected (data not shown). Phorbol esters produced similar results, consistently reducing transcription of exon 1 by 20% and exon 3 by 80%. These data are consistent with the hypothesis that c-myc in HL-60 cells is regulated in part by interruption of transcription after exon 1.

Discussion

The regulation of the proto-oncogene c-myc has been studied in detail in HL-60 cells because expression of this gene, which is high in the basal state, is dramatically reduced by agents that cause differentiation (19-25). In this study, we have extended the work of Kronke et al. (29) and Tobler et al. (36), which showed that the cytokine TNF reduced expression of c-myc. Unexpectedly, IFN did not reduce the expression of c-myc, although IFN is an effective inducer of phenotypic differentiation. Similarly, although IFN was synergistic with TNF in causing phenotypic differentiation, there was no synergism in reduction of c-myc expression. This implies that IFN may induce its effects on HL-60 differentiation by a pathway different from other inducers of differentiation, all of which reduce c-myc expression as a relatively early event. Likewise, the synergy between TNF and IFN may be due to mechanisms other than enhanced regulation of c-myc.

TNF and phorbol esters caused a decline in expression of c-myc when expression was measured with an exon 3 probe. However, dot blots probed for exon 1 revealed a much smaller decline in expression. This suggested that transcriptional regulation occurred at least partly through interruption of transcription after exon 1. Transcription run-on studies confirmed this hypothesis. Tobler, Johnston, and Koeffler have recently demonstrated that TNF regulated c-myc expression at the transcriptional level (36). Their probes for c-myc, however, hybridized only to sequences for exons 2 and 3 and, therefore did not detect transcription interruption occurring between exons 1 and 2. Kronke and co-workers similarly described effects of TNF on expression of c-myc exon 3 but did not address the question of transcription interruption (29).

Bentley and Groudine have investigated transcription interruption in response to retinoic acid, an inducer of myeloid maturation of HL-60 cells, and localized the site of transcription termination to an area near the first exon-intron boundary (31). Chung *et al* have demonstrated that in whole cell extracts termination of transcription by RNA polymerases II and III occurs in

this same region (37). Examination of the sequence in this area reveals a thymidine rich region with several poly-T segments and regions of dyad symmetry potentially capable of forming hairpin loops. Regions capable of forming hairpin loops function in some prokaryotic systems as attenuators or terminators of transcription (38,39). Little is known however, about their role in regulation of eukaryotic transcription termination. Recent evidence in model systems suggests that RNA polymerase II recognizes certain poly-T regions as termination sites without a definite requirement for specific adjacent secondary structure (40). Since TNF, phorbol esters, and retinoic acid enhance termination of RNA polymerase II transcription within a region where termination is partially suppressed in untreated HL-60 cells, it is tempting to hypothesize that these substances alter the function of an antiterminator. This may be a common mechanism of c-myc regulation and further studies may provide insights into general mechanisms of transcriptional control.

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