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Genetics

Induction of differentiation in HL60 cells by the reduction of extrachromosomally amplified c-*myc*

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ABSTRACT **Oncogene amplification in tumor cells results in the overexpression of proteins that confer a growth advantage *in vitro* and *in vivo*. Amplified oncogenes can reside intrachromosomally, within homogeneously staining regions (HSRs), or extrachromosomally, within double minute chromosomes (DMs). Since previous studies have shown that low concentrations of hydroxyurea (HU) can eliminate DMs, we studied the use of HU as a gene-targeting agent in tumor cells containing extrachromosomally amplified oncogenes. In a neuroendocrine cell line (COLO 320), we have shown that HU can eliminate amplified copies of c-*myc* located on DMs, leading to a reduction in tumorigenicity *in vitro* and *in vivo*. To determine whether the observed reduction in tumorigenicity was due to differentiation, we next investigated whether HU could induce differentiation in HL60 cells containing extrachromosomally amplified c-*myc*. We compared the effects of HU, as well as two other known differentiating agents (dimethyl sulfoxide and retinoic acid), on c-*myc* gene copy number, c-*myc* expression, and differentiation in HL60 cells containing amplified c-*myc* genes either on DMs or HSRs. We discovered that HU and dimethyl sulfoxide reduced both c-*myc* gene copy number and expression and induced differentiation in cells containing c-*myc* amplified on DMs. These agents failed to have similar effects on HL60 cells with amplified c-*myc* in HSRs. By contrast, retinoic acid induced differentiation independent of the localization of amplified c-*myc*. These data illustrate the utility of targeting extrachromosomal DNA to modulate tumor phenotype and reveal that both HU and dimethyl sulfoxide induce differentiation in HL60 cells through DM elimination.**

Gene amplification in tumor cells results in the production of multiple copies of a genomic region. Amplification of oncogenes leads to the overexpression of proteins participating in the transduction of growth-related signals and confers a growth advantage to tumor cells (1, 2). Clinically, oncogene amplification is extremely common in human tumors and correlates with a poor prognosis for patients with ovarian cancer (*HER-2/neu*), breast cancer (c-*myc*, *HER-2/neu*), neuroblastoma (N-*myc*), or small cell lung cancer (c-*myc*) (3–6).

Amplified oncogenes can be located on chromosomes, within homogeneously staining regions (HSRs), or they can reside extrachromosomally either as submicroscopic elements called episomes or as larger structures called double minute chromosomes (DMs) (7–9). DMs are paired, acentric fragments that segregate randomly at cell division and can be detected in the majority of primary tumors at biopsy (10, 11). The tendency of DMs to integrate into chromosomes during passage *in vitro* provides one explanation for the variable incidence of DMs in cultured cell lines (8, 10). We and others (12, 13) have shown that the number of DMs increases after cultured cells are grown as tumors in nude mice, suggesting that DMs may encode genes that provide a growth or survival advantage *in vivo*. One such example is the *mdm*-2 gene (cloned from a murine DM), which encodes a protein that binds and inactivates wild-type p53 (14, 15).

Genes amplified on DMs can be lost spontaneously at each cell division or can be eliminated by treatment with hydroxyurea (HU) at concentrations that do not inhibit DNA synthesis or ribonucleotide reductase (13, 16–19). The mechanism of HU-induced DM elimination is mediated by an increase in micronuclei formation, and the capture of the DMs within micronuclei (13, 20). By contrast, HSRs are not lost during cell division or by treatment with HU and represent a "stable" form of gene amplification (13, 17). Since DMs contain amplified genes (known and unknown) and are susceptible to elimination by HU, we have been investigating the use of HU as a gene-targeting agent in cells that contain extrachromosomally amplified c-*myc* genes. Previously, we showed that the elimination of DM-encoded c-*myc* genes from a colon cancer cell line of neuroendocrine origin (COLO 320 DM) reduced its tumorigenicity in nude mice (13).

Since there are many studies suggesting that overexpression of c-*myc* is associated with increased proliferation and reduced ability to differentiate (21, 22), we next pursued an investigation using HU to eliminate extrachromosomally amplified c-*myc* genes in HL60 cells. It seemed likely that elimination of c-*myc*-containing DMs from COLO 320 DM cells had reduced tumorigenicity by inducing differentiation, but it was difficult to analyze this relationship due to the absence of convenient markers of differentiation. By contrast, HL60 cells are easily assessed for differentiation into granulocyte or macrophage lineages and different isolates are available that contain amplified c-*myc* genes either on DMs or HSRs (8, 23).

In this study we show that low concentrations of HU reduce the number of c-*myc*-containing DMs in HL60 cells, which leads to decreased c-*myc* expression and induction of differentiation. Furthermore, dimethyl sulfoxide (DMSO), a well-known polar solvent, also induces differentiation by this mechanism. In contrast to HU and DMSO, retinoic acid (RA) induces differentiation in HL60 cells independent of the localization of amplified c-*myc* genes. These studies further establish the ability of agents that eliminate extrachromosomal DNA to alter tumor phenotype and provide a rational basis for designing innovative therapeutic strategies.

METHODS

Cell Lines. An early passage of the HL60 promyelocytic leukemia cell line, obtained from S. Collins (Fred Hutchinson

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Abbreviations: HU, hydroxyurea; DM, double minute chromosome; HSR, homogeneously staining region; DMSO, dimethyl sulfoxide; RA, retinoic acid.

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Cancer Research Center, Seattle), contains 16–32 copies of the c-*myc* oncogene localized on extrachromosomal DNA ranging from 250-kbp episomes to DMs (8). Passage 67, subclone 173, contains a median of eight DMs per cell and was used for this study (HL60-DM). Passage 133, subclone 173, has approximately the same number of c-*myc* genes integrated into chromosomes (HL60-HSR). Fluorescence *in situ* hybridization with a c-*myc* probe (24) showed that the early HL60-DM passage contained <5% of cells with HSRs, while the late HL60-HSR passage contained <1% spreads with DMs. The HL60-HSR cells served as a control for the effects of HU on cell growth and viability.

HL60 cells were grown in RPMI 1640 medium with 10% fetal bovine serum and 2 mM glutamine. HU (Squibb) was added on day 0 of each culture at the concentrations indicated and was replaced each time the cells were passaged (13). All cells were passaged to maintain a density of <2 million cells per ml. All-*trans*-β-RA (RA) (Sigma) was diluted to a final concentration of 1 μM from a stock of 1 mM in ethanol, such that the final concentration of ethanol was <0.1%. DMSO was added at a final concentration of 1.3% (vol/vol).

Determination of c-*myc* Gene Copy Number and Expression. Genomic DNA was prepared using an SDS lysis technique (25) and the fraction of the initial c-*myc* copy number was determined by dot-blot hybridization to a c-*myc* probe (Oncor). The amount of total DNA was internally controlled by subsequent hybridization to a chicken β-actin probe (Oncor). The ratio of c-*myc* to β-actin was then calculated using a densitometer (Image 1.45) or PhosphorImager (Molecular Dynamics) and compared to the control, to yield relative percent.

Total RNA was prepared using RNAzol (Tel-Test, Friendswood, TX), and the relative amount of c-*myc* expression was quantitated by hybridization with a c-*myc* probe (Oncor). The amount of total RNA loaded was controlled by subsequent hybridization to a chicken β-actin probe (Oncor). The ratio of c-*myc* to β-actin was calculated using a PhosphorImager (Molecular Dynamics) and compared to the control, to yield relative percent.

Differentiation Analyses. Differentiation was assessed using a functional assay for mature myelocytes (23). For nitroblue tetrazolium reduction, 300,000 cells were suspended in 0.2 ml of RPMI 1640 medium supplemented with 10% fetal bovine serum and incubated for 20 min at 37°C with an equal volume of 0.1% nitroblue tetrazolium (Sigma) and 50 ng of freshly diluted phorbol 12-myristate 13-acetate (Sigma). Cytospin slides were prepared and counted for the percentage of cells containing intracellular reduced blue-black formazan deposits by counting at least 500 cells and correcting for viability.

Cloning Efficiency. Cells exposed to HU were washed in RPMI medium and then plated on soft agar at a concentration of 60,000 cells per plate for HL60-HSR or 80,000 cells per plate for HL60-DM cells (26). This resulted in a range of 0–350 colonies per plate.

Evaluation of Cells for Micronuclei and DMs. HL60 cells were exposed to colcemid (0.1 μg/ml; GIBCO) for 1–3 hr, incubated in 0.075 M KCl for 20 min, fixed in methanol/glacial acetic acid (3:1), and dropped onto wet slides. Micronuclei and DMs were counted from preparations of metaphase chromosome spreads and interphase nuclei. Micronuclei were reported per 1000 nuclei, and DMs were averaged from 30 metaphase spreads.

RESULTS AND DISCUSSION

Treatment of HL60-DM Cells with HU Leads to a Reduction in c-*myc* Gene Copy Number. Our previous studies in COLO 320 DM cells showed that low concentrations of HU could eliminate extrachromosomally amplified c-*myc* genes and reduce tumorigenicity (13). We hypothesized that the observed reduction in tumorigenicity was due to decreased c-*myc* expression and induction of differentiation. To investigate whether there was an inverse correlation between c-*myc* amplification and differentiation, we studied the effects of DM elimination on the phenotype of HL60 cells. HL60 cells can be easily assessed for differentiation and isolates are available that contain amplified c-*myc* genes localizing to either DMs (HL60-DM) or HSRs (HL60-HSR) (8, 23).

We first analyzed the HU dose–response of HL60-DM and HL60-HSR cells to determine if extrachromosomally amplified c-*myc* genes could be eliminated. The results shown in Fig. 1 are consistent with previous data in other cell lines and indicate that HU induces the elimination of extrachromosomal, but not chromosomal, amplicons in a concentration- and time-dependent manner. For example, a 14-day exposure to 150 μM HU resulted in a 70% reduction in c-*myc* copy number in HL60-DM cells but not in HL60-HSR cells ($P < 0.001$).

Reduction of c-*myc* Gene Copies in HL60-DM Cells Correlates with the Induction of Differentiation and Loss of *in Vitro* Tumorigenicity. An association between c-*myc* gene expression and differentiation has been suggested by the correlations between c-*myc* oncogene overexpression and suppression of differentiation (22, 27) and by the ability of anti-sense c-*myc* transcripts to induce differentiation (28, 29). Furthermore, many classic differentiating agents are associated with a decrease in expression of c-*myc* (30). We therefore investigated whether reducing c-*myc* copy number increased the fraction of differentiated HL60 cells. We compared the differentiation of HL60-DM and HL60-HSR cells in the presence or absence of HU. Two results emerged from the data shown in Fig. 2. (*i*) The incidence of differentiated cells was significantly higher in the untreated HL60-DM population than in the untreated HL60-HSR population ($P = 0.003$ at 14 days). This was likely due to the spontaneous loss of extrachromosomally amplified c-*myc* during random segregation of DMs. (*ii*) HU treatment increased the number of differentiated HL60-DM cells but had no effect on the HL60-HSR cells. For example, 65% of the HL60-DM cells exposed to 150 μM HU for 14 days had differentiated, compared to 8% of the untreated control ($P = 0.0002$). By contrast, 4% of the HL60-HSR cells treated under identical conditions differentiated, compared with 1% of the untreated control. Although we cannot establish a direct cause-and-effect relationship with this experiment, the HU induction of differentiation did parallel the concentration- and time-

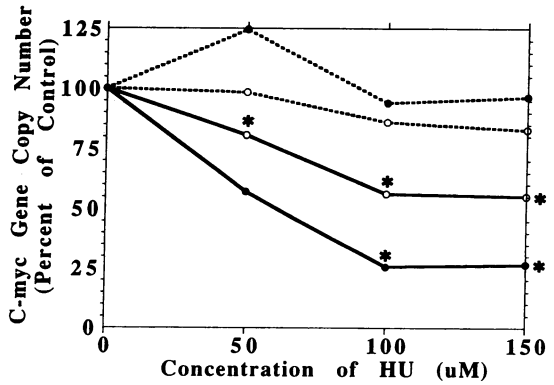

FIG. 1. HU-induced elimination of c-*myc* genes in HL60-DM cells. HL60-DM (solid lines) and HL60-HSR (dashed lines) cells were exposed and processed as described in the text. ○, Seven-day exposure; ●, 14-day exposure. Asterisks represent values in the HL60-DM cells that were significantly different ($P < 0.05$) from the equivalently treated HL60-HSR cells.

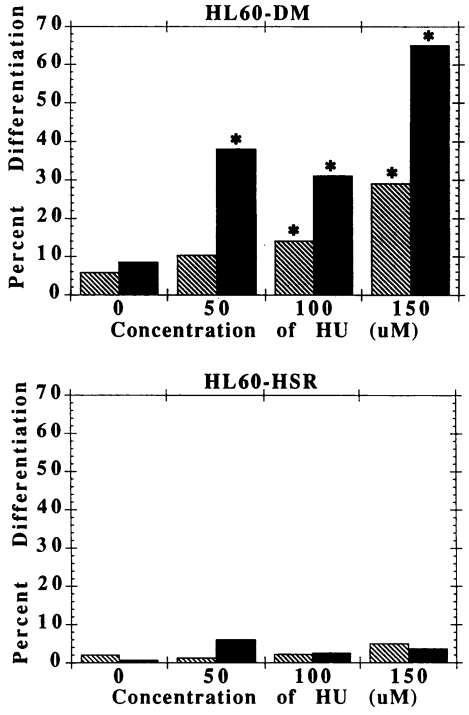

FIG. 2. Induction of differentiation in HL60-DM cells by HU. Both cell lines were treated with indicated concentrations of HU for a total of 14 days. Differentiation was determined at day 7 (hatched bars) and at day 14 (solid bars). Asterisks represent values in the HL60-DM cells that were significantly different ($P < 0.05$) from the equivalently treated HL60-HSR cells.

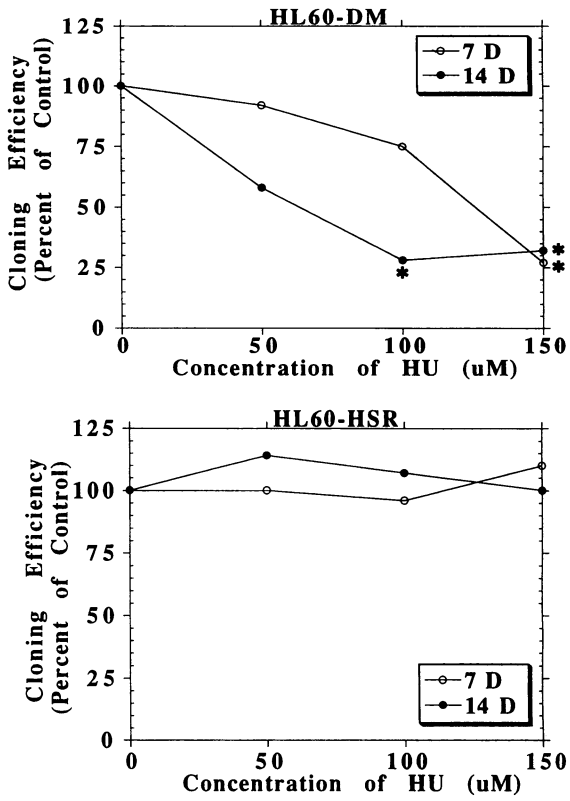

FIG. 3. Reduction in cloning efficiency of HL60-DM cells exposed to HU. Cells were plated according to procedures outlined in the text. Asterisks represent values in the HL60-DM cells that were significantly different ($P < 0.05$) from the equivalently treated HL60-HSR cells.

dependent loss of extrachromosomally amplified c-*myc* genes shown in Fig. 1. This relationship was linear with a correlation coefficient (r) of >0.75. These data demonstrate that the mechanism of HU-induced differentiation is specific for cells containing extrachromosomal amplicons.

Since our previous studies in COLO 320 DM cells revealed an inverse correlation between c-*myc* gene copy number and both *in vitro* and *in vivo* tumorigenicity, we next determined whether HU treatment affected the ability of HL60-DM and HL60-HSR cells to form colonies in soft agar (13). Fig. 3 shows that HU treatment produced a 75% decrease in the cloning efficiency of the HL60-DM cells ($P = 0.014$ at 14 days) but showed no effect on HL60-HSR cells. The time and concentration dependence were similar to that observed for the loss of c-*myc* gene copy number and induction of differentiation. The lack of effect of HU on the cloning efficiency of HL60-HSR cells indicated that the reduction observed in the HL60-DM cells did not result from cytotoxic effects of HU at the concentrations employed.

These results provide strong evidence to support the hypothesis that the reduced tumorigenicity of HU-treated HL60-DM cells derives from reduced oncogene copy number and resulting differentiation.

DMSO and RA Induce Differentiation via Two Distinct Routes. To compare and contrast the effects of HU on HL60 cells with other known differentiating agents, we chose to examine the effects of DMSO and RA on the cells in this system, using the same time points. DMSO is a polar solvent and a nonphysiologic agent that induces differentiation in HL60 and other tumor cells (31). The precise mechanism of action is unknown but has been hypothesized to involve effects on the cellular membrane or on the conformation of DNA (32, 33). Since polar solvents have been reported to induce micronuclei formation (34), we chose to investigate whether DMSO induced differentiation in HL60-DM cells by oncogene elimination. By contrast, RA is a known receptor-mediated, transcriptional regulator capable of inducing differentiation in many systems (35). The available data indicate that RA induces differentiation by direct modulation of gene expression and a rapid down-regulation of c-*myc* (36). We hypothesized that RA would down-regulate c-*myc* gene expression regardless of the localization of amplified genes and induce differentiation equally in HL60-DM and HL60-HSR cells.

Table 1 shows that DMSO produced results similar to HU and that only cells harboring DMs could be induced to differentiate. The mechanism of differentiation involved elimination of extrachromosomally amplified genes, since DMSO produced a reduction in c-*myc* copy number in HL60-DM cells but not in HL60-HSR cells ($P = 0.0006$ at 14 days). The results in Table 1 also show that RA substantially induced differentiation in both HL60-DM and HL60-HSR cells. Furthermore, no significant reduction in c-*myc* copy number was observed in the HL60-DM cells. The fact that RA effectively differentiated the HL60-HSR cells argues

Table 1. Comparison of HU, DMSO, and RA in HL60 cells

Cell type	Drug	% copy number*	% diff.
HL60-DM	HU†	26 ± 2.6	65
	DMSO†	33 ± 7.1	79
	RA‡	81 ± 5.2	82
HL60-HSR	HU†	91 ± 9.6	4
	DMSO†	85 ± 4.0	2
	RA‡	81 ± 3.4	78

Diff., differentiation. Cells were exposed to 150 μM HU, 1.3% DMSO, or 1 μM RA for the times indicated.
*Relative to control.
†Fourteen-day exposure.
‡Seven-day exposure.

against the presence of an alteration in these cells that could have produced an irreversible block to differentiation. These results confirm that RA-induced differentiation is not specific to cells containing extrachromosomal DNA and consistent with a mechanism of transcriptional down-regulation of c-*myc*.

To further investigate the mechanism of differentiation in DMSO- and HU-treated cells, we prepared metaphase spreads of HL60-DM and HL60-HSR cells and counted both the number of micronuclei and the number of DMs. The results are shown in Table 2. Both DMSO and HU caused a reduction in the number of DMs; however, only HU induced the formation of micronuclei. Consistent with our data in COLO 320 cells, there was also some induction of micronuclei by HU in the HL60-HSR cells, but <10% contained c-*myc* DNA when analyzed by fluorescence *in situ* hybridization (data not shown). Thus, DMSO appears to eliminate c-*myc*-containing DMs in HL60-DM cells, but the mechanism of elimination may not be the formation of micronuclei.

Reduced Steady-State c-*myc* Expression Is the Final Common Pathway to Differentiation in HL60 Cells. The data presented above demonstrate that HU and DMSO facilitate the loss of c-*myc*-containing DMs in HL60 cells, leading to the induction of differentiation. Our hypothesis is that HL60-DM cells exist in a dynamic state of DM loss and gain, suggesting that the resulting phenotype (treated or untreated) is dependent on whether the individual cell has lost sufficient copies of c-*myc* to fall below a critical threshold level of c-*myc* expression. The reduction of steady-state c-*myc* expression may be accomplished by decreasing copies of the gene or by down-regulating its expression. This was investigated by analyzing the level of steady-state c-*myc* RNA expression in HL60-DM and HL60-HSR cells treated with HU, DMSO, and RA. Fig. 4 shows that the c-*myc* RNA levels decreased in the HL60-DM cells treated with either HU or DMSO but remained unchanged in the HL60-HSR cells treated in an identical fashion. In combination with the data shown in Fig. 1 and Table 1, these data indicate that the reduction in c-*myc* expression produced by these agents parallels the observed changes in copy number. By contrast, RA decreased c-*myc* RNA levels in both HL60-DM and HSR cells without affecting copy number. As shown in Fig. 5, the reduction in steady-state c-*myc* RNA correlated with an increase in differentiated cells. HU and DMSO required a longer exposure than RA to induce a maximal decrease in steady-state c-*myc* expression and to generate the highest fraction of differentiated cells (data not shown). The prolonged time course of HU and DMSO is consistent with a mechanism of copy number loss through cell division, whereas RA works more rapidly through a direct effect on gene expression.

These data support the evidence that overexpression of c-*myc* can block differentiation (22, 27) and illustrate two

Table 2. Comparison of the effects of HU and DMSO on micronuclei formation and double minutes in HL60-DM and HL60-HSR cells

Cell type	Drug*	No. of micronuclei†	Av. no. of DMs/ metaphase spread‡
HL60-DM	None	8	18
	HU	44	4
	DMSO	9	2
HL60-HSR	None	2	<1
	HU	12	<1
	DMSO	2	<1

Av., average.
*Cells were exposed to 150 μM HU or 1.3% DMSO for 7 days.
†Per 1000 nuclei.
‡Thirty spreads counted.

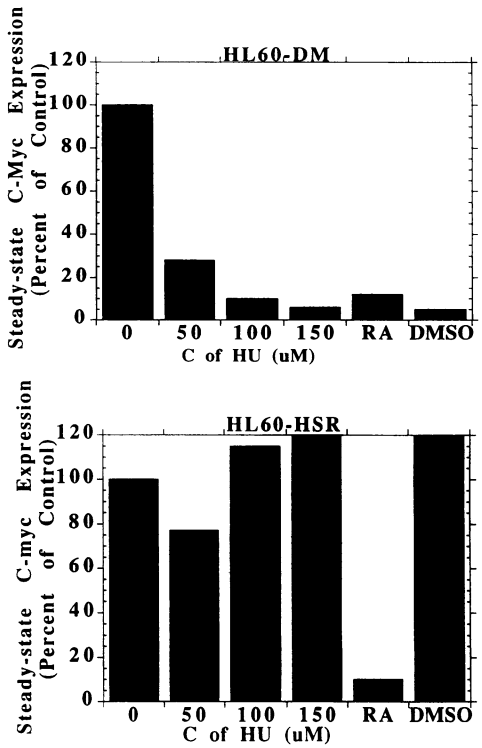

FIG. 4. Steady-state c-*myc* expression in treated HL60-DM and HL60-HSR cells. Cells were exposed and processed as described in the text. Exposure time was 7 days for RA and 14 days for HU and DMSO.

routes to differentiation that can reverse this blockade. We show that DMSO and HU can accelerate the loss of extrachromosomally amplified genes in HL60-DM cells, resulting in reduced c-*myc* expression and induction of differentiation. The lack of effect of these agents on the HL60-HSR cells indicates that the mechanism of DMSO- and HU-induced differentiation is specific to cells containing extrachromosomally amplified genes. In addition, it suggests that the chromosomal integration of amplified genes may be a mechanism whereby resistance to differentiation by this route is generated. By contrast, RA effectively differentiates both HL60-DM and HL60-HSR cells by a mechanism that is independent of the localization of amplified c-*myc*. Consistent with these results is a previous report showing synergistic effects on differentiation in HL60 cells with combinations of RA and either DMSO, hexamethylene bisacetamide, or sodium butyrate (37). Our data provide a rationale to explain this observed synergism by showing that RA and DMSO function through distinct routes to induce differentiation in cells containing extrachromosomally amplified c-*myc*. Reduction in c-*myc* gene expression is the common feature of these routes to differentiation, indicating that oncogene overexpression is a significant factor in determining the phenotype of HL60 cells.

In summary, the demonstration that HU induces differentiation and reduces the cloning efficiency of HL60-DM cells is consistent with our initial hypothesis in COLO 320 DM cells. By studying the phenotypic effects of reduced copies of extrachromosomally amplified c-*myc* genes in COLO 320 and HL60 cells, we have established a rationale for using HU as a gene-targeting agent in cells harboring amplified oncogenes (known or unknown) on DMs. In addition, we have characterized two other known differentiating agents with respect to their effects on extrachromosomal DNA. The finding that HU and RA function through two different pathways to reduce c-*myc* expression in HL60-DM cells provides a scientific basis for investigating the use of these agents in

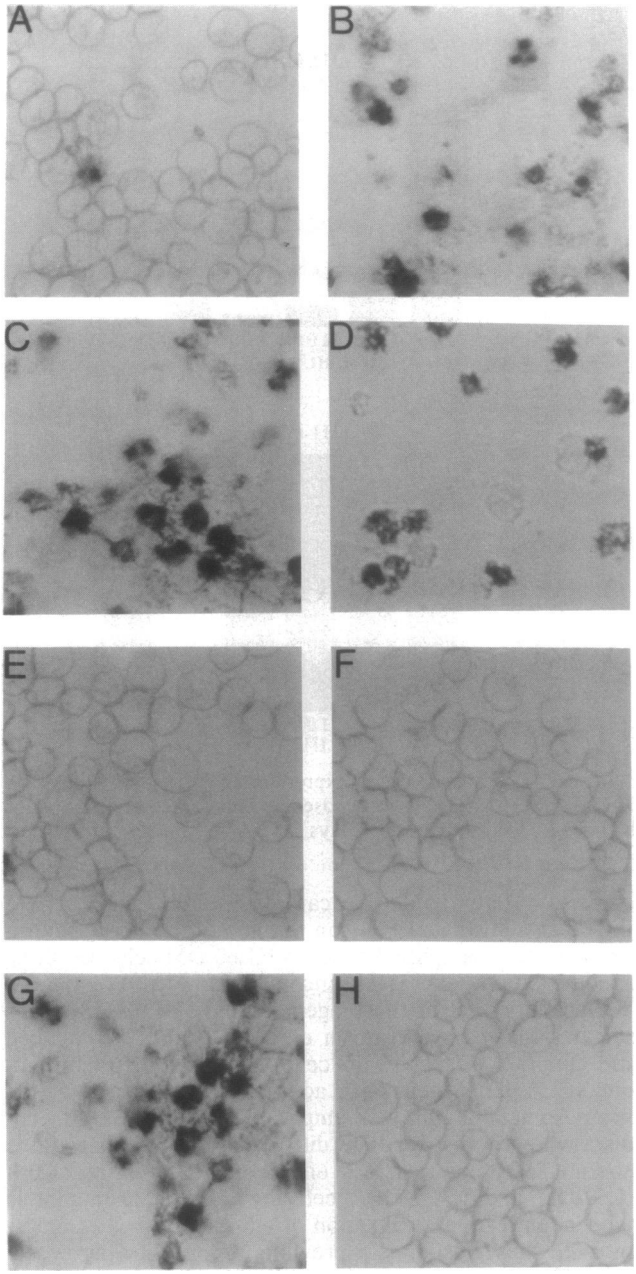

FIG. 5. Phenotype of treated HL60-DM and HL60-HSR cells. The nitroblue tetrazolium assay was performed as described in the text. (*A–D*) HL60-DM cells. (*E–H*) HL60-HSR cells. *A* and *E*, untreated. *B* and *F*, 150 mM HU. *C* and *G*, RA. *D* and *H*, DMSO. Exposure time was 7 days for RA and 14 days for HU and DMSO. (×26.)

combination in cells containing extrachromosomally amplified oncogenes.

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