MOLECULAR AND CELLULAR BIOLOGY, May 1986, 1698–1705
0270-7306/86/051698-08$02.00/0

Vol. 6, No. 5

Gamma Interferon and 5-Azacytidine Cause Transcriptional Elevation of Class I Major Histocompatibility Complex Gene Expression in K562 Leukemia Cells in the Absence of Differentiation

EMILY CHEN,[1,2] ROBERT W. KARR,[2] JOAN P. FROST,[2] THOMAS A. GONWA,[2] AND GORDON D. GINDER[1,2]*

Veterans Administration Medical Center and Department of Internal Medicine, University of Iowa College of Medicine,[2] and Genetics Ph.D. Program, University of Iowa,[1] Iowa City, Iowa 52242

Received 13 December 1985/Accepted 19 February 1986

We studied the effects of gamma interferon (IFN-γ) on HLA class I gene expression, differentiation, and proliferative capacity of K562 human leukemia cells. In the uninduced state, K562 cells show little or no class I gene expression but actively express the erythroid-specific γ-globin gene as well as genes associated with cell proliferation, including the transferrin receptor, c-*myc*, and α-actin genes. At both the surface protein and mRNA levels, IFN-γ induces class I and β_2-microglobulin gene expression, but does not alter the expression of the γ-globin, transferrin receptor, c-*myc*, or α-actin genes. A 10-fold maximal induction of both class I surface protein and mRNA occurs at 48 h and is reversible upon withdrawal of IFN-γ from the culture medium. In vitro nuclear run-on transcription assays were performed to directly establish that IFN-γ exerts an early effect at the level of transcription, with maximal transcription rates occurring within 4 h. The difference between the time course of transcription induction and that of mRNA accumulation suggests that the regulation of class I gene expression in this human leukemic cell line also involves posttranscriptional mechanisms. Measurements of cell proliferation rates and cell cycle distribution, as well as the reversibility of the effects of IFN-γ, demonstrate that the selective induction of class I genes in these cells occurs in the absence of differentiation.

The human major histocompatibility complex consists of a large family of genes which encode cell surface molecules involved in immune regulation and function. Since HLA class I and class II antigens are involved in recognition of non-self and immune responsiveness to virally infected cells and are associated with certain disease states, modulation of HLA expression could have dramatic biologic effects. There is increasing evidence that the oncogenicity of certain human and animal tumors is closely associated with the degree of class I gene expression. This is felt to be related to the ability of tumor cells with altered class I expression to evade the immune system (7, 44, 49, 51).

Interferons have been reported to induce major histocompatibility complex gene expression and to cause differentiation in several hematopoietic cell lines (3, 4, 37, 41, 48), in normal hematopoietic cells (15, 26), in nonhematopoietic transformed cell lines (6, 12, 38), and in vivo in patients with lung cancer (36). In other studies it has been shown that class I genes can be induced by gamma interferon (IFN-γ) in neuroblastoma cell lines without shifting the population towards more differentiated neuronal forms (29). While the increased steady-state HLA class I mRNA levels reported in many cases are consistent with a transcriptional effect, this has not been directly demonstrated.

The K562 human cell line, which was derived from the pleural effusion of a patient with chronic myelogenous leukemia in terminal blast crisis (31), is one of several leukemic cell lines used as model systems to study hematopoietic cell differentiation and gene expression. Most sublines of K562 cells can be induced to differentiate via the erythroid pathway by more than 20 agents (32, 43). A noteworthy feature of this cell line is that it has markedly decreased or absent class I gene expression.

We used the K562 cell line as a model to study the effects of IFN-γ on class I gene expression and differentiation. The purposes of these studies were (i) to determine at what level IFN-γ induces class I antigen expression in the K562 cell line and (ii) to determine whether IFN-γ alone or in combination with 5-azacytidine (5-Aza) could discernibly alter the state of differentiation in K562 cells. We found from immunofluorescence and Northern blot mRNA quantitation that IFN-γ alone increases class I antigen expression by fivefold and that the addition of 5-Aza results in a further twofold increase. In vitro nuclear transcription run-on assays were carried out to directly establish for the first time that IFN-γ exerts a regulatory effect on class I gene expression at the transcriptional level. However, kinetic analyses demonstrated a disparate time course for maximal class I gene transcriptional rate compared with stable cytoplasmic mRNA levels, thus suggesting that IFN-γ also exerts part of its regulatory effect at the posttranscriptional level. In contrast to class I genes, the mRNA levels of the erythroid-specific γ-globin gene, as well as other genes which are usually regulated during differentiation, remained unchanged. In addition, neither the cell cycle position nor the proliferative capacity of the total cell population was modified by the IFN-γ treatment employed in these experiments. Our results indicate that IFN-γ alone or in combination with 5-Aza does not alter the state of differentiation in the K562 cell line used in these studies, but that this agent is capable of selectively inducing expression of specific genes, such as

* Corresponding author.

the class I heavy chain and β_2-microglobulin (β_2m) genes, at the transcriptional level.

MATERIALS AND METHODS

Cells. The K562 cell line was obtained from J. Minowada and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, glutamine, and gentamicin (complete medium). The cell line was documented to be free of mycoplasma.

Reagents. Propidium iodide, 5-Aza, and RNase were purchased from Sigma Chemical Co. (St. Louis, Mo.). Affinity-purified human IFN-γ was obtained from Interferon Sciences, Inc. (New Brunswick, N.Y.). Fluorescein-conjugated $F(ab')_2$ fragments of goat anti-mouse immunoglobulin G were obtained from Cooper Biomedical, Inc. (West Chester, Pa.).

Monoclonal antibodies. The 5E9 monoclonal antibody (MAb) detects the human transferrin receptor (22). The W6/32 MAb detects a nonpolymorphic determinant on the heavy chain of human class I molecules (5). The BBM.1 MAb is specific for human β_2m (9). The 5E9, W6/32, and BBM.1 hybridomas were obtained from the American Type Culture Collection, Rockville, Md. SG157 is an MAb (obtained from S. Goyert) that is specific for HLA-DR molecules (19). The Leu-10 MAb (Becton Dickinson, Mountain View, Calif.) is specific for HLA-DQ molecules.

Culture conditions. K562 cells were cultured at 3×10^5 to 4×10^5 cells per ml in complete medium with the following additives: (i) 5-Aza only, 2 μM; (ii) IFN-γ only, 100 U/ml; (iii) 5-Aza + IFN-γ; and (iv) no additives (referred to as uninduced). In the experiments to study the decrement of the induced changes after removal of the additives, samples of cells were removed from each culture condition after 48 h, washed twice in complete medium, and cultured at 5×10^5 cells per ml in complete medium without additives in new cell culture flasks. Samples of cells cultured in this manner were collected after 4, 24, 48, and 144 h for analysis.

Flow cytometric analysis. The surface immunofluorescence and DNA content of individual cells were analyzed by flow cytometry with log amplification utilizing a fluorescence-activated cell sorter (FACS IV, Becton Dickinson). Samples of 2×10^6 cells to be analyzed for surface immunofluorescence were first incubated with human immunoglobulin for 30 min at 4°C to decrease nonspecific binding of the MAbs to Fc receptors. The cells were washed once, incubated with saturating amounts of various MAbs or the mouse immunoglobulin-negative control, followed by fluorescein-conjugated goat anti-mouse immunoglobulin G, and analyzed by flow cytometry as previously described (18). The median channel fluorescence (MCF) reported for the experimental samples represents the MCF of the sample minus the MCF of the appropriate control. Under saturating conditions, fluorescence intensity as measured by MCF is an indication of antigen density on the cell surface (35). In this system, a change of MCF equal to 66 channels represents a log-fold difference in intensity. Cells for simultaneous surface immunofluorescence and DNA content (cell cycle) analysis using propidium iodide were prepared as described by Kehrl et al. (25). For each sample, 30,000 cells were analyzed. The percentage of positive cells and MCF with each MAb were determined separately for cells in the G_0/G_1, S, and G_2/M phases of the cell cycle, based on propidium iodide staining.

RNA isolation. RNA was isolated from 10^7 to 10^8 cells by a rapid nuclear-cytoplasmic fractionation method conducted at 4°C using diethylpyrocarbonate-treated reagents. Cells were washed in NTM buffer (140 mM NaCl, 10 mM Tris chloride, pH 8.4, 1.5 mM $MgCl_2$) in Microfuge tubes and spun in a Microfuge at 12,000 × *g* for 10 s. The pellets were suspended in 2 ml of NTM buffer–0.5% Nonidet P-40–250 U of RNasin (Promega Biotec) per ml, and the suspensions were spun in a Microfuge for 20 s. Immediately the supernatants (cytoplasmic fraction) were removed to fresh Microfuge tubes, and sodium dodecyl sulfate (SDS) was added to a final concentration of 0.3%. The RNA was extracted with neutral phenol and chloroform, ethanol precipitated, and stored at −20°C until ready for use. Poly(A)$^+$ mRNA was isolated by oligo(dT)-cellulose column (Bethesda Research Laboratories) chromatography as described by Maniatis et al. (34).

Quantitation of mRNA. Poly(A)$^+$ RNA, measured spectrophotometrically at 260 nm, was glyoxylated by the method of Thomas (50) and loaded at approximately 5 μg per lane on 1% agarose gels. Electrophoresis, transfer to nitrocellulose (Schleicher & Schuell; 0.45-μm pore size), and hybridization to ^{32}P-labeled nick-translated probes were as previously described (17). Washes were at 65°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS–5 mM sodium pyrophosphate for 15 min and 0.1× SSC–0.1% SDS for 30 min. For rehybridizations, old probe was melted off the filters in 10 mM Tris chloride (pH 8.0)–1 mM EDTA–50% formamide at 65°C for 2 h. This treatment removed 95 to 100% of the bound probe.

Probes used for quantitation of mRNA. The following cloned human DNAs were used as probes: (i) HLA-B7, 1.4-kilobase (kb) cDNA from clone pDP001 (46); (ii) β_2m, 0.55-kb cDNA (47); (iii) HLA-DRα, 3.1-kb genomic DNA from clone pP34-RI-3 (11); (iv) α-actin, 1.5-kb cDNA from clone pHMαA-1 (21); (v) transferrin receptor, 4.9-kb cDNA from clone pCD-TR1 (28); (vi) γ-globin, 1.1-kb cDNA from clone JW151 (52); (vii) pE3-*myc*, 1.7-kb *Cla*I-*Eco*RI genomic DNA fragment from clone λ hR1-15 (42).

Nuclear run-on transcription assay. Nuclear transcription and RNA isolation were performed with the following modifications of published procedures (8, 20). IFN-γ- and 5-Aza-treated K562 cells were washed twice with RPMI 1640 containing no fetal bovine serum and twice with 0.32 M sucrose–2 mM $MgCl_2$–1 mM sodium phosphate (pH 6.8). The cells were suspended in 10 mM NaCl–1 mM sodium phosphate (pH 6.8), spun at 800 × *g* for 10 min, suspended in 0.32 M sucrose–1 mM $MgCl_2$–0.04% Triton X-100–1 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4), and lysed in a Dounce homogenizer with five strokes using pestle A. The mixture was spun at 800 × *g* for 10 min and washed twice with RB (0.1 M NaCl, 50 mM Tris chloride, pH 8.0, 3 mM $MgCl_2$, 5 mM butyrate, 0.1 mM phenylmethylsulfonyl fluoride). Nuclei were suspended in RB at a concentration of 40 μl of RB and 40 μl of glycerol per 10^8 cells and stored at −80°C. Transcription reactions (200 μl, total volume) contained 10^8 nuclei, 30% glycerol, 80 U of RNasin, 2.5 mM dithiothreitol, 1 mM $MgCl_2$, 70 mM KCl, 10 mM phosphocreatine, 0.25 mM each UTP and CTP, 0.5 mM ATP, and 250 μCi of [α-^{32}P]GTP (Amersham; 410 Ci/mmol). Transcripts were deproteinized, DNase I treated, and further purified by chromatography on a G-50 Sephadex spin column (8, 20). Aqueous hybridization to Southern blot Nytran (Schleicher & Schuell) filters containing 3 to 5 μg of HLA-B7 or α-actin cDNA insert in each lane was at 65°C for 48 h. Filters were washed first in 2× SSC–0.1% SDS for 30 min at 65°C and then in 0.1× SSC–0.1% SDS for 30 min at 65°C. Filters were further washed in 2× SSC, 20 μg of

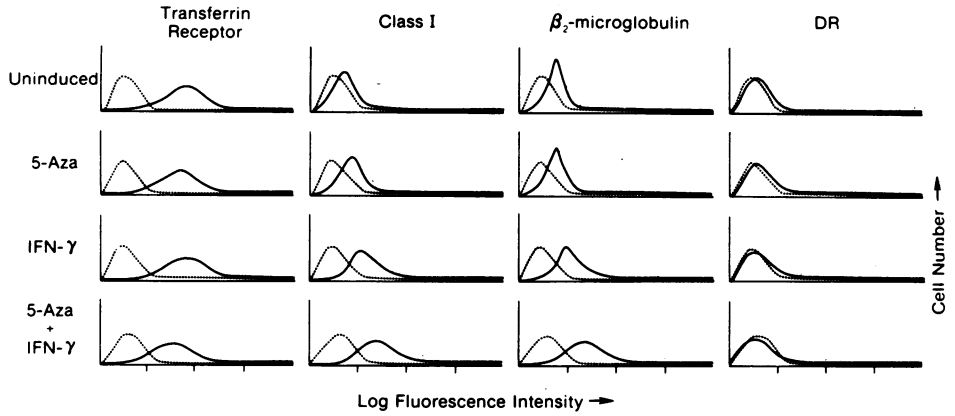

FIG. 1. Fluorescence histograms of IFN-γ + 5-Aza induction of transferrin receptor, class I, β_2m, and DR antigens. Nonspecific staining with the negative control antibody is shown as a dotted line. Specific staining with each MAb is shown as a solid line. The corresponding MCF values are given in Table 1.

pancreatic RNase A per ml, and 8 U of RNase T_1 per ml for 30 min at 37°C, in 0.1× SSC–0.1% SDS–50 μg of proteinase K per ml for 30 min at 37°C, and then in 0.1× SSC–0.1% SDS for 1 to 2 h at 65°C.

Cell proliferation analysis. At the times indicated during the cultures, samples of cells were removed for determination of cell number, viability, and proliferative capacity. Cell number was determined using a Coulter counter (Coulter Electronics, Hialeah, Fla.), and viability was determined by trypan blue exclusion. Proliferative capacity was assayed by [^{3}H]thymidine incorporation. Samples of cells (2×10^5) in 200 μl of the medium in which they had been cultured were placed in round-bottom 96-well microtiter plates (Costar). One microcurie of [^{3}H]thymidine (Amersham; specific activity, 5 Ci/mmol) was added to each well, and plates were incubated for 4 h at 37°C in 95% air–5% CO_2. All assays were performed in triplicate. At 4 h, plates were harvested onto fiberglass filters by standard techniques and [^{3}H]thymidine incorporation was measured by counting in a liquid scintillation counter. Results are presented as counts per minute per 10^5 viable cells.

RESULTS

IFN-γ and 5-Aza additively induce HLA class I and β_2m surface expression and mRNA levels. K562 cells were cultured, and the cell surface expression of the transferrin receptor, class I molecules, β_2m, and the DR and DQ HLA class II molecules was analyzed by indirect immunofluorescence and flow cytometry as described in Materials and Methods. The fluorescence histograms of a representative experiment are shown in Fig. 1; the percentage of K562 cells positive with each MAb and the corresponding MCF values for multiple experiments are given in Table 1. Uninduced K562 cells expressed the transferrin receptor at high levels: 94% of the cells were positive with an MCF of 83. In contrast, uninduced K562 cells expressed very low levels of class I molecules, β_2m, and DR and DQ molecules. After culture in 2 μM 5-Aza for 48 h, K562 cells continued to express the transferrin receptor at high levels. In most experiments, the expression of class I molecules and β_2m increased slightly in the 5-Aza-treated cells; in contrast, there was no change in the DR or DQ expression with 5-Aza. IFN-γ produced a marked increase in the expression of class I molecules and β_2m: over 50% of the cells were positive for class I and β_2m, with corresponding increases in the MCFs. IFN-γ produced no change in the expression of DR or DQ molecules or the transferrin receptor. Interestingly, the combination IFN-γ + 5-Aza induced the greatest increase in class I and β_2m expression by K562 cells: 78% were class I positive, and 84% were β_2m positive. There was also a corresponding increase in the MCF values for both class I and β_2m compared with the values for IFN-γ alone. On the other hand, the addition of 5-Aza to IFN-γ caused no change in expression of the transferrin receptor or DR or DQ molecules. Therefore, our results show that IFN-γ induces expression of class I molecules and β_2m on the K562 cell surface, in agreement with a recent report (48). In addition, these data document that the combination of IFN-γ + 5-

TABLE 1. Flow cytometric analysis of cell surface antigen expression

Antigen	MAb	Treatment							
		Uninduced		5-Aza		IFN-γ		5-Aza + IFN-γ	
		% Positive[a]	MCF[b]	% Positive[a]	MCF[b]	% Positive[a]	MCF[b]	% Positive[a]	MCF[b]
Transferrin receptor[c]	5E9	94 ± 0.5	83 ± 2.0	93 ± 1.0	94 ± 20.4	95 ± 0.5	99 ± 14.5	89 ± 5.0	89 ± 27.0
Class I[d]	W6/32	5 ± 2.6	3 ± 3.3	18 ± 6.1	18 ± 5.2	57 ± 11.1	33 ± 5.5	78 ± 6.7	57 ± 5.0
β_2m[d]	BBM.1	17 ± 7.0	9 ± 9.0	25 ± 4.5	17 ± 5.0	56 ± 15.0	31 ± 7.5	84 ± 2.0	54 ± 1.5
DR[d]	SG157	1 ± 0.6	0	2 ± 2.3	2 ± 2.0	1 ± 1.0	0	2 ± 1.5	4 ± 2.2
DQ[c]	Leu-10	1 ± 1.0	0	3 ± 3.0	3 ± 3.0	1 ± 1.0	2 ± 1.5	4 ± 3.5	5 ± 4.5

[a] Percent cells positive with given MAb ± standard error.
[b] MCF for cells positive with given MAb ± standard error.
[c] Two experiments.
[d] Three experiments.

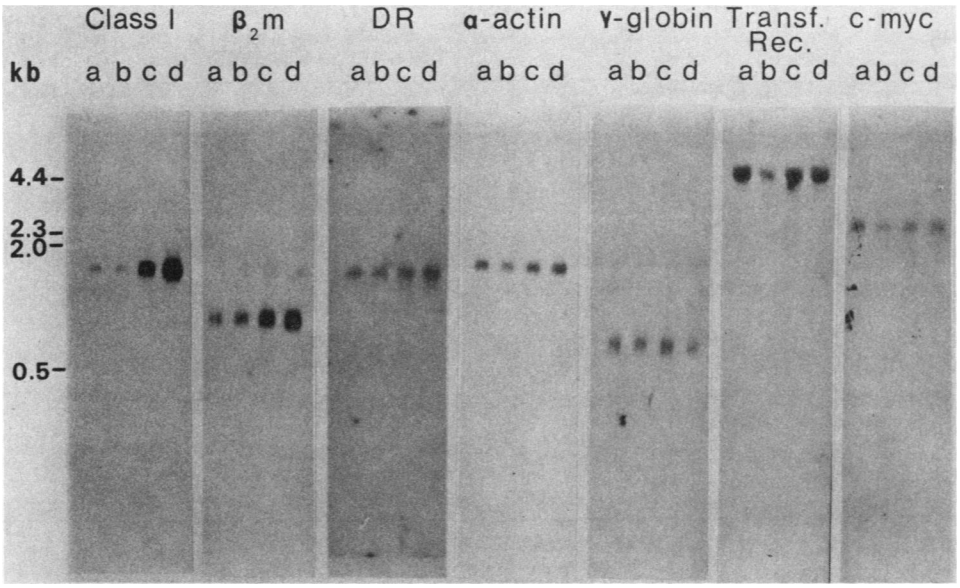

FIG. 2. mRNA quantitation by Northern blotting. A sample of 5 μg of glyoxylated poly(A)$^+$ mRNA was loaded in each lane. Each panel is the same filter, sequentially hybridized to seven probes as indicated. Treatments: lanes a, uninduced; lanes b, 5-Aza (48 h); lanes c, IFN-γ (48 h); lanes d, 5-Aza + IFN-γ (48 h). The corresponding densitometric quantitation of band intensities is given in Table 2.

Aza produces an increase that exceeds the effect of IFN-γ alone.

Northern blotting was performed to determine whether mRNA levels correlate with cell surface expression to rule out possible translational or posttranslational regulation. Figure 2 shows that class I, HLA-DRα, and β_2m mRNAs were induced by 5-Aza and IFN-γ, whereas no induction was seen for α-actin, γ-globin, transferrin receptor, or c-*myc* mRNAs. Quantitatively, IFN-γ alone caused an increase in mRNA levels of 4.9-fold for class I genes, 2.4-fold for the β_2m gene, and 1.5-fold for the HLA-DRα gene (Table 2). In combination with 5-Aza, IFN-γ caused a 10-fold increase for class I genes, a 3-fold increase for β_2m genes, and a 2-fold increase for the HLA-DRα gene. It is interesting that both the mRNA level and the surface expression (MCF) for class I genes were increased by IFN-γ + 5-Aza by about a log-fold (Table 1), whereas the mRNA level for β_2m did not increase in exact proportion to the surface immunofluorescence. The constant γ-globin mRNA level is consistent with the absence of differentiation either via the erythroid pathway, which should be accompanied by increased globin RNA, or via a nonerythroid pathway, which should be accompanied by decreased globin RNA.

In the Friend erythroleukemia, U937, and HL-60 cell lines, there is a close link between reduction of c-*myc* expression and G_0/G_1 arrest when either phorbol myristate acetate or dimethyl sulfoxide is used to trigger terminal differentiation (14). The lack of down-regulation of c-*myc* by IFN-γ in K562 cells in our studies is most consistent with a lack of terminal differentiation. The constant transferrin receptor mRNA level is consistent with the observation that the rate of cell proliferation is constant under all conditions studied, as shown in Table 4 and discussed below.

Kinetics of class I gene induction as determined by immunofluorescence, Northern blotting, and nuclear run-on transcription assays. To gain further insight into the possible levels of class I gene regulation in this system, we examined the kinetics of class I induction at the levels of protein, stable steady-state mRNA, and transcription rate. K562 cells were induced with 100 U of IFN-γ per ml plus 2 μM 5-Aza for 48 h (2 days) before the two agents were removed from culture for the following 144 h (6 days). The class I surface expression and mRNA levels began to rise within 24 h of induction, peaked at 48 h, and dropped to near the uninduced levels by 144 h after 5-Aza + IFN-γ were withdrawn (Fig. 3). The same time course was observed in cells treated with IFN-γ alone (data not shown). Induction, therefore, is reversible, which is consistent with selective modulation of class I genes in the absence of true differentiation. The transferrin receptor gene was used as a negative, noninducible control, and the lack of change in its expression reflects the constant rate of cell division and the availability of adequate iron in the medium throughout the 8-day time course.

The observations that the class I surface expression and mRNA levels continue to rise even after 24 h yet do not exactly parallel one another suggested that both transcriptional and posttranscriptional mechanisms may be involved

TABLE 2. Relative intensities of Northern hybridization bands[a]

Band	Treatment: Uninduced	5-Aza (2 days)	IFN-γ (2 days)	5-Aza + IFN-γ (2 days)
Class I (HLA-B7)	1.0	0.9	4.9	10.0
β_2m	1.0	1.3	2.4	3.0
Class II (HLA-DRα)	1.0	1.3	1.5	1.9
α-Actin	1.0	0.9	1.1	1.3
γ-Globin	1.0	0.9	1.1	0.8
Transferrin receptor	1.0	0.4	1.0	0.8
c-*myc*	1.0	0.6	0.7	0.6

[a] Autoradiograms were scanned at 550 nm on a Beckman DU-8 absorbance scanner. Triplicate peaks were integrated by cutting out and weighing on an analytical balance. For a given probe, all intensities are relative to the uninduced K562 cell band intensity, which is assigned the value 1.0.

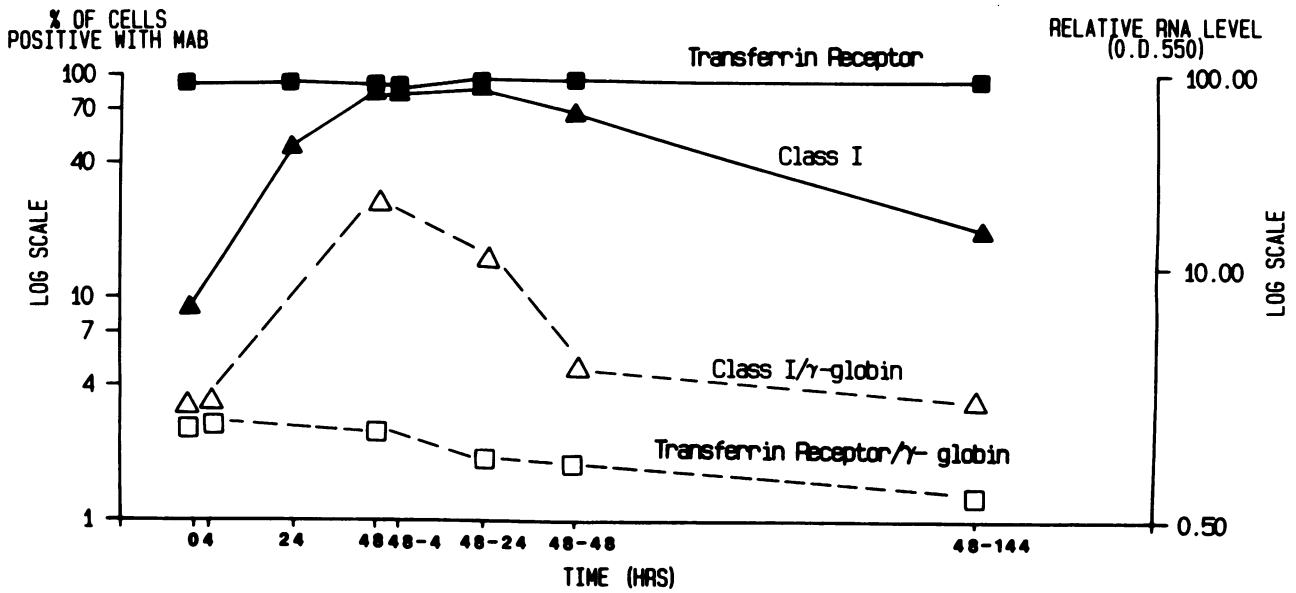

FIG. 3. Kinetics of class I and transferrin gene expression in IFN-γ + 5-Aza-treated K562 cells. Solid lines, immunofluorescence intensities; dashed lines, mRNA levels. The numbers 0, 4, 24, and 48 on the horizontal scale refer to the number of hours for which the cells were continually grown in 5-Aza + IFN-γ. At 48 h, the two agents were removed from culture. The numbers 48-4, 48-24, 48-48, and 48-144 refer to the number of hours after the 48-h time point in which the cells were cultured without the agents. For mRNA quantitation, scanning densitometry was performed on autoradiograms of a filter which was sequentially hybridized to the HLA-B7, transferrin receptor, and γ-globin probes. The relative mRNA level was calculated by dividing the value obtained from scans for HLA-B7 and transferrin genes by the value obtained from scans for the γ-globin gene at each time point. This was done to correct for variable amounts of RNA loaded in each lane; γ-globin was arbitrarily chosen as the denominator since it is not induced by 5-Aza or IFN-γ.

in class I gene regulation in this model. To directly test this hypothesis, in vitro run-on transcription assays using nuclei isolated at various time points of IFN-γ + 5-Aza treatment were performed. No class I transcripts were detectable in uninduced cells (0 h) (Fig. 4). Since some class I mRNA is detectable in these cells, this most likely indicates a very low amount of class I transcription which is below the level of sensitivity of our assay. By 30 min of IFN-γ + 5-Aza treatment, class I transcripts could be detected, and the level became maximal by 4 h. The increased class I transcription level at 4 h was most likely caused by IFN-γ only, since no transcripts were detected when nuclei isolated from cells treated for 4 h with 5-Aza alone were assayed (data not shown). The high level of class I transcription continued at 48 h of IFN-γ + 5-Aza treatment. For each assay performed, transcription of class I (Fig. 4, lanes a) and α-actin (Fig. 4, lanes b) was measured. The α-actin transcription levels were measured to correct for any technical variability between assays, assuming that the level of transcription of α-actin is constant throughout the 48-h time course, which seems a reasonable assumption given the constant level of α-actin mRNA at all time points.

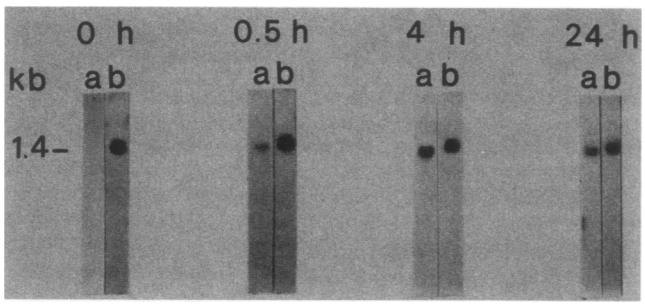

FIG. 4. Nuclear run-on transcription assay using nuclei isolated after 0, 0.5, 4, and 24 h of IFN-γ + 5-Aza treatment. Transcripts were hybridized to filters containing cDNAs as described in Materials and Methods. Lanes a, HLA-B7 cDNA; lanes b, α-actin cDNA.

IFN-γ and 5-Aza induction do not alter cell cycle position nor cell proliferation rate. One original aim of our studies was to determine the effect of IFN-γ and 5-Aza on K562 cell differentiation. Previous studies have suggested that a major mode of action of both of these agents involves induction of cell differentiation and that specific genes, including that for HLA, are activated as a part of a differentiation program (24, 48). The exact mechanisms of action of IFN-γ are not well understood (30), but it has been shown to exert cytostatic or cytotoxic effects. 5-Aza is a cytotoxic drug with pleiotropic effects on cells, including inhibition of cytosine methyltransferase (10), induction of certain genes, and modulation of cell surface antigen expression and metastatic behavior (40). Therefore, one possible mechanism by which these agents may act alone or in concert could involve selective toxicity, resulting in the enrichment of a subpopulation of cells which already manifest high levels of class I gene expression or are spontaneously entering a state of differentiation characterized by high class I gene expression. Alternatively, these agents could shift the distribution of cells to the G_0 or G_1 phase of the cell cycle as a part of the process of commitment to differentiation (14). To rule out these possible mechanisms, we examined the effect of IFN-γ and 5-Aza on cell cycle position and cell proliferation. Table 3 shows the results of a comparison of cell cycle position and cell surface expression of the transferrin receptor, class I molecules, and β_2m, using flow cytometry. Importantly, the percentages of cells in each phase of the cell cycle that were positive for class I or β_2m were very similar within each culture condition. In addition, the percentage of cells in each phase of the

TABLE 3. Comparison of cell cycle position and immunofluorescence

Antigen	MAb	Cell cycle phase	Treatment: Uninduced % Positive[a]	Uninduced MCF[b]	Uninduced % Total cells[c]	5-Aza % Positive[a]	5-Aza MCF[b]	5-Aza % Total cells[c]	IFN-γ % Positive[a]	IFN-γ MCF[b]	IFN-γ % Total cells[c]	5-Aza- + IFN-γ % Positive[a]	5-Aza- + IFN-γ MCF[b]	5-Aza- + IFN-γ % Total cells[c]
Transferrin receptor	5E9	G_0/G_1	93	52	43	88	44	30	93	62	40	85	44	24
		S	94	47	34	91	44	39	92	52	39	85	44	45
		G_2/M	94	44	23	92	44	31	92	49	21	89	45	31
Class I	W6/32	G_0/G_1	18	6	40	18	8	28	76	26	41	86	42	29
		S	22	7	40	18	8	40	71	21	39	81	37	44
		G_2/M	20	5	20	25	9	32	73	19	20	91	38	27
β_2m	BBM.1	G_0/G_1	16	6	40	17	8	31	81	27	40	84	40	28
		S	15	6	38	15	7	37	74	22	38	77	34	43
		G_2/M	13	4	22	20	9	32	78	20	22	90	35	29

[a] Percent cells positive with given MAb.
[b] MCF for cells positive with given MAb.
[c] Percent total cells in indicated cell cycle phase.

cell cycle did not reflect G_0/G_1 arrest as would be expected in terminal differentiation (14).

The lack of cytotoxic cell selection was corroborated by determination of the number of viable K562 cells and the cell proliferation rates as measured by [^{3}H]thymidine incorporation. In a representative experiment (Table 4), cell viability and [^{3}H]thymidine incorporation did not change with any of the various culture conditions as compared with the uninduced control cells. Therefore, by these parameters, there is no evidence that IFN-γ, with or without 5-Aza, either selects a subpopulation of cells which already expresses high levels of the class I genes or induces or selects for differentiation.

DISCUSSION

We have found that induction of HLA class I genes in K562 cells can occur as a separate phenomenon from any discernible program of differentiation. IFN-γ alone or in combination with 5-Aza induces class I gene expression, but does not induce the expression of γ-globin, c-*myc*, α-actin, or transferrin receptor genes. While it is possible that at higher and most likely toxic concentrations of IFN-γ and 5-Aza some degree of cell differentiation might occur, the induction of class I gene expression in these studies appears to be a direct and selective modulation. We have also shown by nuclear run-on assays that IFN-γ exerts at least part of its effect at a transcriptional level. Results of recently reported studies of class I gene regulation in a human cell line treated with IFN-α are consistent with the involvement of both transcriptional and posttranscriptional mechanisms (16). However, given the fact that IFN-γ binds to a different receptor than IFN-α or -β (30), and given the delayed time course of class I transcriptional activation shown here compared with that observed in studies of IFN-α and -β (16), it is reasonable to expect that the mechanisms of class I gene regulation by these different types of interferons may be quite distinct. In support of this possibility, indirect analyses of 2′,5′-oligo(A) polymerase mRNA induction have also shown a delayed time course for IFN-γ compared to IFN-α or -β (2). On the other hand, it has been demonstrated that IFN-γ can induce rapid transcriptional activation of at least one gene in the U937 cell line (33). This finding, along with the delayed time course of transcription induction seen in our studies, raises the possibility that the effect of IFN-γ on class I gene transcription may require, or be mediated by, the product of some early response gene. Alternatively, the delayed time course of induction of class I transcription described here could reflect a mechanism in which IFN-γ down-regulates a labile repressor. These possibilities are currently under study.

In these studies, β_2m expression paralleled class I expression, although induction at the mRNA level by IFN-γ + 5-Aza was greater for class I (10-fold) than for β_2m (3-fold). At the surface level, class I increased 10-fold and β_2m increased 6-fold. One explanation for this difference in the class I heavy chain/β_2m ratio for mRNA versus the surface protein is that there are β_2m molecules that are not associated with HLA heavy chains on the surface. It has been shown that on lymphocyte cell surfaces, there may be 10-fold as many β_2m molecules as HLA molecules (1).

Exactly what role 5-Aza played in inducing class I expression in our experiments is not yet known, but since the effect is additive with IFN-γ, it presumably acts by a separate mechanism. 5-Aza is known to alter the differentiation state of certain cells (39), to modulate certain aspects of the tumor phenotype and antigen expression, and to selectively induce

TABLE 4. Analysis of proliferation by [^{3}H]thymidine incorporation

Treatment	0 h Viable cells per ml (10^5)	0 h [^{3}H]thymidine cpm[a]	24 h Viable cells per ml (10^5)	24 h [^{3}H]thymidine cpm[a]	48 h Viable cells per ml (10^5)	48 h [^{3}H]thymidine cpm[a]
Uninduced	3.0	95,355 ± 11,465	6.9	83,918 ± 3,383	14.0	66,312 ± 1,937
5-Aza	4.0		5.0	65,527 ± 6,042	11.0	89,018 ± 2,789
IFN-γ	3.0		4.9	101,194 ± 5,889	14.0	79,397 ± 1,663
5-Aza + IFN-γ	4.0		4.6	81,840 ± 9,227	12.0	73,507 ± 5,305

[a] Per 10^5 viable cells.

certain genes; most of these effects are felt to be due to demethylation of DNA (23, 24). Even at the low, nontoxic drug concentrations used here, 5-Aza treatment results in demethylation of the cytosines in the DNA of particular genes, and may in so doing alter the ability of any *trans*-acting factors which are induced by IFN-γ to affect class I gene transcription. When DNA from K562 cells was digested with *Hpa*II and *Msp*I followed by Southern blot analysis using the HLA-B7 probe, we observed that some of the methylated CCGG sites in the class I gene cluster that were methylated in uninduced K562 DNA became demethylated after IFN-γ + 5-Aza treatment but not after treatment with IFN-γ alone (data not shown). However, these results are difficult to interpret since the HLA-B7 probe used in these studies cross-hybridizes with a multitude of class I-like genes (46), so that specific sites in the complex Southern blot band patterns cannot be precisely mapped to determine whether they lie in expressed or nonexpressed gene sequences. As reported in our previous studies of embryonic globin gene regulation in vivo (17) and as reviewed elsewhere (23), demethylation by 5-Aza is not confined to actively expressed genes, and thus it is often not sufficient to cause gene activation. Studies are presently under way to identify which HLA class I genes are expressed in induced K562 cells, to determine whether 5-Aza treatment results in demethylation or induction, or both, of additional class I type genes which are not affected by IFN-γ treatment alone, or whether it merely increases the expression of IFN-induced genes.

The reason for low class I gene expression in K562 cells is as yet unknown. Like the K562 cell line, certain human neuroblastoma, small-cell lung carcinoma, and lung adenocarcinoma cell lines have been reported to express low levels of class I genes (12, 29). This has been associated with amplified levels of a c-*myc*-related oncogene, N-*myc* (12). In our experiments, c-*myc* mRNA detected by the c-*myc* probe was not elevated, even at low hybridization stringency, which should detect related N-*myc* expression (data not shown). Therefore, low class I gene expression in the K562 cell line does not appear to be related to cellular *myc* proto-oncogene expression. An alternative cause for the low class I gene expression observed in these cells may be the presence of another cellular gene product analogous to the E1A gene product of adenovirus 12, which has been shown to control the level of MHC expression in some cell lines transformed with that virus. This effect of the adenovirus 12 E1A product can be reversed by IFN-γ treatment (7, 13). Such a factor could act by a *trans*-acting blocking effect (27), and current technology should allow this hypothesis to be tested. On the other hand, low class I gene expression in K562 cells may result from the absence of a positive regulatory factor. Finally, in light of the indirect evidence for posttranscriptional regulation of HLA class I expression in K562 cells presented here, it is possible that part of the effect resides at the posttranscriptional level.

In summary, we have shown that in K562 human leukemia cells, IFN-γ with or without the addition of 5-Aza selectively induces class I gene expression in a dose-dependent way in the absence of discernible differentiation. While we have directly established that IFN-γ, like IFN-α and -β, is a potent inducer of class I gene transcription, our kinetic analyses indicate that the mechanism by which IFN-γ induces transcription of class I genes may be different from that for INF-α or -β. Studies are under way to analyze further the mechanisms of regulation of class I gene activation by IFN-γ in human leukemia cells, as well as to determine the relationship between class I gene expression and oncogenicity.

ACKNOWLEDGMENTS

We thank S. Goyert, A. McClelland, P. Gunning, L. Kedes, K. Itakura, J. Pan, S. Weissman, H. Kazazian, M. Erisman, and S. Astrin for kindly providing us with probes. We also thank M. Whitters for doing all the nick translations, and J. House, J. Wilkerson, and T. Farmer for their help in preparation of the manuscript.

E.C. is a graduate student in Genetics in the Medical Scientist Training Program. R.W.K. and G.D.G. are Clinical Investigators and T.A.G. is a Research Associate of the Veterans Administration. This work was supported by grant 1730 from the Council for Tobacco Research U.S.A., Inc., to R.W.K. and T.A.G. and by Public Health Service grant AM29904 from the National Institutes of Health to G.D.G.

LITERATURE CITED

1. **Apella, E., and J. A. Sawicki.** 1984. β_2-Microglobulin. Methods Enzymol. **108**:494–504.
2. **Baglioni, C., and P. A. Maroney.** 1980. Mechanism of action of human interferons: induction of 2′5′-oligo(A) polymerase. J. Biol. Chem. **255**:8390–8393.
3. **Ball, E. D., P. M. Guyre, J. M. Glynn, W. F. C. Rigby, and M. W. Fanger.** 1984. Modulation of class I HLA antigens on HL-60 promyelocytic leukemia cells by serum-free medium: reinduction by γ-IFN and 1,25-dihydroxyvitamin D_3 (calcitriol). J. Immunol. **132**:2424–2428.
4. **Ball, E. D., P. M. Guyre, L. Shen, J. M. Glynn, C. R. Maliszewski, P. E. Baker, and M. W. Fanger.** 1984. Gamma interferon induces monocytoid differentiation in the HL-60 cell line. J. Clin. Invest. **73**:1072–1077.
5. **Barnstable, C. J., W. F. Bodmer, G. Brown, G. Galfre, C. Milstein, A. F. Williams, and A. Ziegler.** 1978. Production of monoclonal antibodies to group A erythrocytes, HLA, and other human cell surface antigens—new tools for genetic analysis. Cell **14**:9–20.
6. **Basham, T. Y., and T. C. Merigan.** 1983. Recombinant interferon-γ increases HLA-DR synthesis and expression. J. Immunol. **130**:1492–1494.
7. **Bernards, R., P. I. Schrier, A. Houweling, J. L. Bos, A. J. Van der Eb, M. Zijlstra, and C. J. M. Melief.** 1983. Tumorigenicity of cells transformed by adenovirus type 12 by evasion of T-cell immunity. Nature (London) **305**:776–779.
8. **Bitter, G. A., and R. G. Roeder.** 1978. Transcription of viral genes by RNA polymerase II in nuclei isolated from adenovirus 2 transformed cells. Biochemistry **17**:2198–2205.
9. **Brodsky, F. M., W. F. Bodmer, and P. Parham.** 1979. Characterization of a monoclonal anti-β_2-microglobulin antibody and its use in the genetic and biochemical analysis of major histocompatibility antigens. Eur. J. Immunol. **9**:536–545.
10. **Christman, J. K., N. Hendelsohn, D. Herzog, and N. Schneiderman.** 1983. Effect of 5-azacytidine on differentiation and DNA methylation in human promyelocytic leukemia cells (HL-60). Cancer Res. **43**:763–769.
11. **Das, H. K., P. A. Biro, S. N. Cohen, H. A. Erlich, A. von Gabain, S. K. Lawrence, P. G. Lemaux, H. O. McDevitt, B. M. Peterlin, M. Schulz, A. K. Sood, and S. M. Weissman.** 1983. Use of synthetic oligonucleotide probes complementary to genes for human HLA-DR α and β as extension primers for the isolation of 5′-specific genomic clones. Proc. Natl. Acad. Sci. USA **80**:1531–1535.
12. **Doyle, A., W. J. Martin, K. Funa, A. Gazdar, D. Carney, S. E. Martin, I. Linnoila, F. Cuttitta, J. Mulshine, P. Bunn, and J. Minna.** 1985. Markedly decreased expression of class I histocompatibility antigens, protein, and mRNA in human small cell lung cancer. J. Exp. Med. **161**:1135–1151.
13. **Eager, K. B., J. Williams, D. Breiding, S. Pan, B. Knowles, E. Appella, and R. P. Ricciardi.** 1985. Expression of histocompatibility antigens H-2K, -D, and L is reduced in adenovirus-12-transformed mouse cells and is restored by interferon γ. Proc. Natl. Acad. Sci. USA **82**:5525–5529.

14. **Einat, M., D. Resnitzky, and A. Kimchi.** 1985. Close link between reduction of c-myc expression by IFN and G_0/G_1 arrest. Nature (London) **313**:597–600.
15. **Fellous, M., U. Nir, D. Wallach, G. Merlin, M. Rubinstein, and M. Revel.** 1982. Interferon-dependent induction of mRNA for the major histocompatibility antigens in human fibroblasts and lymphoblastoid cells. Proc. Natl. Acad. Sci. USA **79**:3082–3086.
16. **Friedman, R. L., S. P. Manly, M. McMahon, I. M. Kerr, and G. R. Stark.** 1984. Transcriptional and posttranscriptional regulation of interferon-induced gene expression in human cells. Cell **38**:745–755.
17. **Ginder, G. D., M. W. Whitters, and J. K. Pohlman.** 1984. Activation of a chicken embryonic globin gene in adult erythroid cells by 5-azacytidine and sodium butyrate. Proc. Natl. Acad. Sci. USA **81**:3954–3958.
18. **Gonwa, T. A., and J. D. Stobo.** 1984. Differential expression of Ia molecules by human monocytes. J. Clin. Invest. **74**:859–866.
19. **Goyert, S. M., J. E. Shively, and J. Silver.** 1982. Biochemical characterization of a second family of human Ia molecules, HLA-DS, equivalent to murine I-A subregion molecules. J. Exp. Med. **156**:550–556.
20. **Groudine, M., M. Peretz, and H. Weintraub.** 1981. Transcriptional regulation of hemoglobin switching in chicken embryos. Mol. Cell. Biol. **1**:281–288.
21. **Gunning, P., P. Ponte, H. Okayama, J. Engel, H. Blau, and L. Kedes.** 1983. Isolation and characterization of full-length cDNA clones for human α-, β-, and γ-actin mRNAs: skeletal but not cytoplasmic actins have an amino-terminal cysteine that is subsequently removed. Mol. Cell. Biol. **3**:787–795.
22. **Haynes, B. F., M. Hemler, T. Cotner, D. L. Mann, G. S. Eisenbarth, J. L. Strominger, and A. S. Fauci.** 1981. Characterization of a monoclonal antibody (5E9) that defines a human cell surface antigen of cell activation. J. Immunol. **127**:347–351.
23. **Jones, P. A.** 1985. Altering gene expression with 5-azacytidine. Cell **40**:485–486.
24. **Jones, P. A., and S. M. Taylor.** 1980. Cellular differentiation, cytidine analogs and DNA methylation. Cell **20**:85–93.
25. **Kehrl, J. H., A. Muraguchi, and A. S. Fauci.** 1984. Differential expression of cell activation markers after stimulation of resting human B lymphocytes. J. Immunol. **132**:2857–2861.
26. **Koeffler, H. P., J. Ranyard, L. Yelton, R. Billing, and R. Bohman.** 1984. γ-Interferon induces expression of the HLA-D antigens on normal and leukemic human myeloid cells. Proc. Natl. Acad. Sci. USA **81**:4080–4084.
27. **Kingston, R. E., A. S. Baldwin, and P. A. Sharp.** 1985. Transcription control by oncogenes. Cell **41**:3–5.
28. **Kuhn, L. C., A. McClelland, and F. H. Ruddle.** 1984. Gene transfer, expression and molecular cloning of the human transferrin receptor gene. Cell **37**:95–103.
29. **Lampson, L. A., and C. A. Fisher.** 1984. Weak HLA and β_2-microglobulin expression of neuronal cell lines can be modulated by interferon. Proc. Natl. Acad. Sci. USA **81**:6476–6480.
30. **Lebleu, B., and J. Content.** 1982. Mechanisms of interferon action: biochemical and genetic approaches, p. 47–94. *In* I. Gresser, (ed.), Interferon 4. Academic Press, Inc., New York.
31. **Lozzio, C. B., and B. B. Lozzio.** 1975. Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. Blood **45**:321–334.
32. **Luisa-DeLuca, C., T. Mitchell, D. Spriggs, and D. W. Kufe.** 1984. Induction of terminal differentiation in human K562 erythroleukemia cells by arabinofuranosylcytosine. J. Clin. Invest. **74**:821–827.
33. **Luster, A. D., J. C. Unkeless, and J. V. Ravetch.** 1985. γ-Interferon transcriptionally regulates an early-response gene containing homology to platelet proteins. Nature (London) **315**:672–676.
34. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
35. **Muirhead, K. A., T. C. Schmitt, and A. R. Muirhead.** 1983. Determination of linear fluorescent intensities from flow cytometric data accumulated with logarithmic amplifiers. Cytometry **3**:251–256.
36. **Nissen, M. H., T. Plesner, J. K. Larsen, B. K. Olesen, and P. Ernst.** 1985. Enhanced expression *in vivo* of HLA-ABC antigens and β_2-microglobulin on human lymphoid cells induced by human interferon-α in patients with lung cancer. Enhanced expression of class I major histocompatibility antigens prior to treatment. Clin. Exp. Immunol. **59**:327–335.
37. **Perussia, B., E. T. Dayton, V. Fanning, P. Thiagarajan, J. Hoxie, and G. Trinchieri.** 1983. Immune interferon and leukocyte-conditioned medium induce normal and leukemic myeloid cells to differentiate along the monocytic pathway. J. Exp. Med. **158**:2058–2080.
38. **Pfizenmaier, K., H. Bartsch, P. Scheurich, B. Seliger, U. Ucer, K. Vehmeyer, and G. A. Nagel.** 1985. Differential γ-interferon response of human colon carcinoma cells: inhibition of proliferation and modulation of immunogenicity as independent effects of γ-interferon on tumor cell growth. Cancer Res. **45**:3503–3509.
39. **Pinto, A., V. Attadia, A. Fusco, F. Ferrara, O. A. Spada, and P. P. Di Fiore.** 1984. 5-Aza-2′-deoxycytidine induces terminal differentiation of leukemic blasts from patients with acute myeloid leukemias. Blood **64**:922–929.
40. **Pinto, A., M. Maio, V. Attadia, S. Zappacosta, and R. Cimino.** 1984. Modulation of HLA-DR antigens expression in human myeloid leukemia cells by cytarabine and 5-aza-2′-deoxycytidine. Lancet **ii**:867–868.
41. **Ralph, P., P. E. Harris, C. J. Punjabi, K. Welte, P. B. Litcofsky, M.-K. Ho, B. Y. Rubin, M. A. S. Moore, and T. A. Springer.** 1983. Lymphokine inducing terminal differentiation of the human monoblast leukemia line U937: a role for γ-interferon. Blood **62**:1169–1175.
42. **Rothberg, P. G., M. D. Erisman, R. E. Diehl, U. G. Rovigatti, and S. M. Astrin.** 1984. Structure and expression of the oncogene c-*myc* in fresh tumor material from patients with hematopoietic malignancies. Mol. Cell. Biol. **4**:1096–1103.
43. **Rowley, P. T., B. M. Ohlsson-Wilhelm, B. A. Farley, and S. LaBella.** 1981. Inducers of erythroid differentiation in K562 human leukemia cells. Exp. Hematol. **9**:32–37.
44. **Sanderson, A. R., and P. C. L. Beverley.** 1983. Interferon, β-2-microglobulin, and immunoselection in the pathway to malignancy. Immunol. Today **4**:211–213.
45. **Schrier, P. I., R. Bernards, R. T. M. J. Vaessen, A. Houweling, and A. J. van der Eb.** 1983. Expression of class I major histocompatibility antigens switched off by highly oncogenic adenovirus 12 in transformed rat cells. Nature (London) **305**:771–775.
46. **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 oligonucleotide primer. Proc. Natl. Acad. Sci. USA **78**:616–620.
47. **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 β_2-microglobulin. Proc. Natl. Acad. Sci. USA **78**:6613–6617.
48. **Sutherland, J., P. Mannoni, F. Rosa, D. Huyat, A. R. Turner, and M. Fellous.** 1985. Induction of the expression of HLA Class I antigens on K562 by interferons and sodium butyrate. Human Immunol. **12**:65–73.
49. **Tanaka, K., K. J. Isselbacher, G. Khoury, and G. Jay.** 1985. Reversal of oncogenesis by the expression of a major histocompatibility complex class I gene. Science **228**:26–30.
50. **Thomas, P. S.** 1983. Hybridization to denatured RNA transferred or dotted to nitrocellulose paper. Methods Enzymol. **100**:255–266.
51. **Wallich, R., N. Bulbuc, G. J. Hammerling, S. Katzav, S. Segal, and M. Feldman.** 1985. Abrogation of metastatic properties of tumour cells by *de novo* expression of H-2K antigens following H-2 gene transfection. Nature (London) **315**:301–305.
52. **Wilson, L. B., J. K. deRiel, L. Villa-Komaroff, A. Efstratiadis, B. G. Forget, and S. M. Weissman.** 1978. Insertion of synthetic copies of human globin genes into bacterial plasmids. Nucleic Acids Res. **5**:563–581.