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Negative and Positive Regulation of Human Leukocyte Antigen Class I Gene Transcription in K562 Leukemia Cells

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The mechanism of transcriptional activation of human leukocyte antigen class I genes by gamma interferon and 5-azacytidine was studied in K562 human leukemia cells. Nuclear run-on transcription assays with various protein and RNA synthesis inhibitors yield evidence for both stimulation of a positive regulatory factor and inhibition of an mRNA that codes for a labile repressor. A novel mechanism is proposed to explain how 5-azacytidine can activate repressed genes without affecting DNA methylation.

The expression of class I human leukocyte antigens (HLAs) encoded by the major histocompatibility complex appears to be important in controlling the metastatic growth of certain murine tumors. Transfection of a class I gene into highly tumorigenic cell lines transformed by adenovirus type 12 or AKR leukemia cell lines that express no detectable class I surface antigens results in expression of class I surface antigen and complete loss of oncogenicity (14, 30, 35, 36). Administration of interferon to mice that have received a tumorigenic dose of adenovirus type 12-transformed class I-deficient cells induces class I expression and results in protection of immunocompetent host animals from the tumor (13). Although such direct relationships between class I expression and oncogenicity have not been demonstrated in human tumors in vivo, many human tumors are known to have deficiencies in class I antigens (8, 18, 31). It has been reported that adenovirus type 12 E1A proteins can repress class I expression in transformed human embryonic cells and that treatment with gamma interferon (IFN-γ) overrides this repressive effect (13, 32). An understanding of the factors that control class I antigen expression in human tumor cells may have therapeutic significance in light of the key role of class I antigens in cytotoxic lymphocyte reactions (6, 11) and recent strategies involving lymphocyte-mediated killing (26).

In this laboratory, the K562 human leukemia cell line (20), which expresses very low levels of class I antigens, has been used as a model to study the molecular mechanisms for decreased class I expression by tumor cells. We reported previously that two agents, IFN-γ and 5-azacytidine (5azaCR), are capable of synergistically increasing class I expression in K562 cells in the absence of differentiation and that at least part of the effect is transcriptional (4). To understand the molecular mechanism of the enhancement of transcription effected by IFN-γ and 5azaCR and the cause of repression of class I transcription in this tumor cell, we have conducted a detailed analysis of the action of these agents. The results allow us to propose a hypothesis that describes the basis of decreased HLA class I gene transcription in K562 cells and accounts for the synergistic effects of 5azaCR and IFN-γ in modulating class I transcription. The mechanism of action of 5azaCR in these experiments is also of general relevance to the interpretation of results in other experimental systems in which 5azaCR has been demonstrated to affect specific gene expression either directly or in combination with other inducers (15).

Class I transcriptional activity in K562 cells treated with 5azaCR or IFN-γ, singly or in combination, was measured by nuclear run-on transcription assays (4). Nuclei were isolated from cells treated with 5azaCR (2 μM) or IFN-γ (100 U/ml), or both agents simultaneously, for the indicated periods between 0 and 48 h. The maximum level of transcription was reached by about 4 h and remained elevated for at least 48 h, as long as both IFN-γ and 5azaCR were present in the medium. Little or no transcription was detected when either agent was present alone (Fig. 1A and B and 2). These results indicate that IFN-γ and 5azaCR synergistically induced class I transcription and that most of the increase in class I steady-state mRNA detected in cells treated with IFN-γ alone in our previous studies (4) was due to effects of IFN-γ on RNA processing or stability.

One striking feature of the time course of class I induction by 5azaCR plus IFN-γ (depicted in Fig. 2) is the relatively long delay (4 h) required for the maximum transcription rate to be reached, compared to the much shorter induction times (<30 min) required for other receptor-mediated transcriptional events, including that reported for interferons (10, 19, 21). This delay could be explained by a requirement for synthesis of an early response protein that increases class I transcription. To test this possibility, transcription induction was carried out in the presence of cycloheximide (CHX), a protein synthesis inhibitor. Adding CHX (50 μg/ml; Sigma Chemical Co.) to the medium 2 h before and during treatment with 5azaCR plus IFN-γ for 4 h completely blocked class I transcription without affecting actin gene transcription (Fig. 1D). Transcription was also blocked by first incubating the cells with CHX for 2 h and then adding 5azaCR plus IFN-γ for up to 24 h (data not shown). Continuous protein synthesis was therefore required for stimulation of class I gene transcription in this tumor cell line, supporting the idea that an early response gene coding for a positive *trans*-acting protein was induced by IFN-γ or 5azaCR plus IFN-γ. This conclusion is also consistent with previous evidence that IFN-γ does not stimulate accumulation of stable mRNA for 2′,5′-oligo(A) polymerase in the presence of protein synthesis inhibitors (1). However, a simple model in which IFN-γ and 5azaCR directly stimulate a positive *trans*-acting factor, which in turn stimulates class I transcription, does not account for the apparent lack of stimulation by either agent alone.

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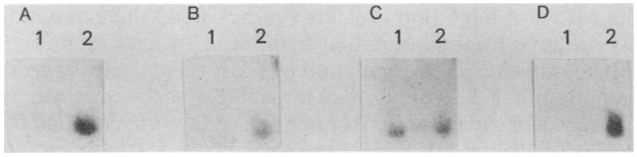

FIG. 1. Transcription of HLA class I and α-actin genes in nuclei from cells treated with various inducers and inhibitors. ^{32}P-labeled transcripts were hybridized to filters containing HLA-B7 class I cDNA sequences (lane 1 in each panel) and human α-actin sequences (lane 2 in each panel) and subjected to autoradiography. The cell treatment regimens corresponding to the transcription assays are as follows: (A) 4 h of IFN-γ (100 U/ml) only; (B) 4 h of 5azaCR (2 μM) only; (C) 4 h of 5azaCR plus IFN-γ; (D) 2 h of CHX (50 μg/ml) followed by 4 h of CHX plus 5azaCR plus IFN-γ.

There is ample evidence that IFN-γ can stimulate HLA gene expression (9, 34), but the role of 5azaCR, especially during the short time course of the present experiments, is not immediately apparent. The major known mechanisms of action of 5azaCR include inhibition of DNA methylation and inhibition of RNA and DNA synthesis after incorporation of 5azaCR into cellular RNA and DNA (33). Previous studies from this laboratory have documented that 5azaCR, at the concentrations used here, does not lead to a significant alteration in DNA synthesis (4), and the 4-h time course required for maximum transcriptional induction by 5azaCR plus IFN-γ would not be consistent with 5azaCR having an effect on DNA synthesis. Similarly, because most studies have shown that the effect of 5azaCR on DNA methylation requires DNA replication (15, 16), hypomethylation of class I genes would also appear to be an unlikely mechanism in this setting. However, a recent report indicates that some chemical inducers can cause rapid and widespread demethylation of DNA in murine erythroleukemia cells within 8 to 12 h (25). To test whether 5azaCR acts to stimulate transcription by inducing rapid DNA demethylation in K562 cells, we assayed the methylation status of CCGG and GCGC sites within HLA class I genes by analysis with the restriction enzymes *Hpa*II, *Hha*I, and *Msp*I. DNA was

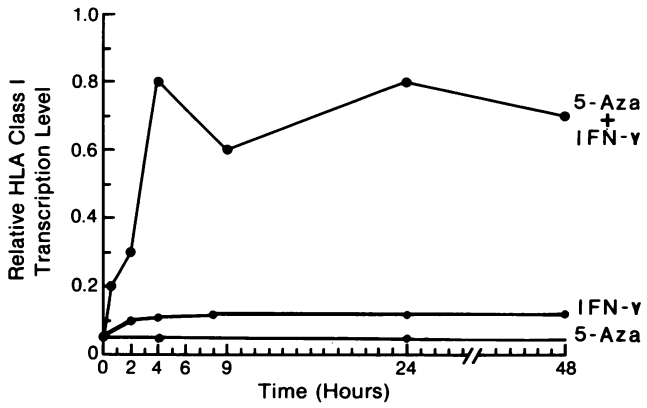

FIG. 2. Transcription time course for K562 cells induced by 5azaCR (5-Aza), IFN-γ, or 5azaCR plus IFN-γ. Autoradiograms such as those in Fig. 1 were scanned at an optical density at 550 nm with a model DU-8 integrating scanning densitometer (Beckman). All HLA class I transcription levels are expressed relative to the transcription levels in untreated cells. Each point on the graph represents an average value derived from at least three separate experiments in which actin transcription was simultaneously measured to control for assay variability.

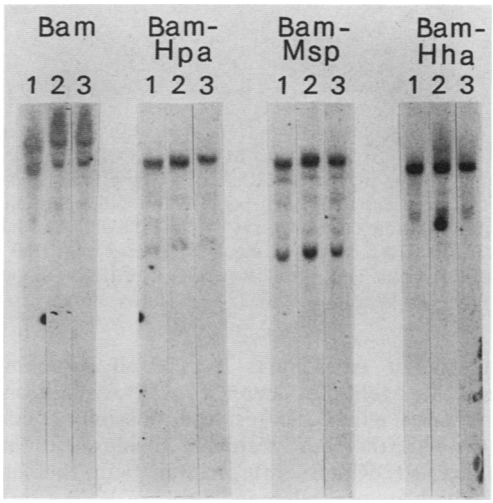

FIG. 3. Southern blot showing the methylation status at CCGG and GCGC sites in the HLA B locus (in each panel) in uninduced cells (lane 1), cells treated with 5azaCR for 4 h (lane 2), and cells treated with 5azaCR plus IFN for 4 h (lane 3). Genomic DNAs were first digested with *Bam*HI (panel 2, lanes 1 to 3), then with *Hpa*II (panel 3, lanes 1 to 3), *Msp*I (panel 4, lanes 1 to 3), or *Hha*I (panel 5, lanes 1 to 3).

isolated from either control cells or cells treated for 4 h with 5azaCR alone or 5azaCR plus IFN-γ. This DNA was analyzed for methylation in the class I gene locus by digestion with either *Hha*I or *Hpa*II, both of which are inhibited by methylation at GCGC or CCGG sites, respectively, or with *Msp*I, which cleaves despite methylation at CCGG sites (22). The resulting Southern blot filter was hybridized to an end-labeled oligonucleotide that is specific for the HLA class I B locus gene (7), which is both expressed and induced by 5azaCR plus IFN-γ treatment (data not shown). The results of this assay (Fig. 3) show that methylation in the vicinity of the HLA class I B locus gene was not changed by treatment of cells with either 5azaCR or 5azaCR plus IFN-γ. The same lack of change in methylation at CCGG and GCGC sites after 4 h of treatment with 5azaCR was observed when the filter shown was rehybridized either to a probe for the human *ApoA*-I gene, which is not expressed in these cells (3), or to the HLA-B7 cDNA probe which hybridizes to multiple expressed class I alleles (27) (data not shown). Thus, extensive strand-symmetric demethylation did not appear to be responsible for the effect of 5azaCR in our experiments, since at the time when maximal transcription was observed, no gross changes in HLA class I gene methylation were detectable.

Given that alterations in DNA synthesis and DNA methylation cannot account for the ability of 5azaCR to synergistically augment class I transcription, the most likely possibility remaining is that 5azaCR acts by inhibiting mRNA synthesis. To explore this possibility, another cytidine analog, cytosine arabinonucleoside (Ara-C), was substituted for 5azaCR in identical induction experiments. Like 5azaCR, Ara-C acts at the S phase of the cell cycle and has been reported to inhibit RNA (5) and DNA synthesis (17), but, unlike 5azaCR, it has little or no effect on DNA methylation (16). Run-on transcription assays from nuclei of cells treated for 4 h with Ara-C (1 μM; Sigma) alone showed no class I transcription (Fig. 4A), but when cells were treated with Ara-C in combination with IFN-γ for 4 h, transcription was stimulated (Fig. 4B).

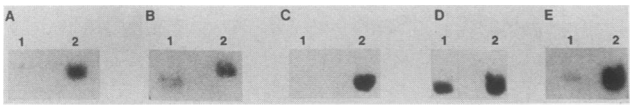

FIG. 4. Effects of Ara-C and DRB on transcription of class I genes. Labeled transcripts were hybridized to Zeta Probe blotting membranes containing class I HLA-B7 (lane 1 in each panel) and α-actin cDNAs (lane 2 in each panel). The cell treatments corresponding to the transcription assay are as follows: (A) 4 h of Ara-C (1 μM) alone; (B) 4 h of Ara-C plus IFN-γ; (C) 2 h of DRB (90 μM); (D) 2 h of DRB plus IFN-γ followed by 2 h of IFN-γ alone; (E) 2 h of DRB plus IFN-γ followed by 22 h of IFN-γ alone.

If 5azaCR or Ara-C acts by partially blocking RNA synthesis, any agent that decreases mRNA synthesis should have the same effect. To test this possibility, cells were cultured with either cordycepin or dichloro-ribofuranosylbenzimidazole (DRB; Boehringer Mannheim Biochemicals), both of which are reversible inhibitors of RNA synthesis (1, 29). As shown in Fig. 4C, after 2 h of DRB (90 μM) treatment, no class I transcription was detected. However, when cells were treated with DRB plus IFN-γ for 2 h, washed to remove the DRB, and then exposed again to IFN-γ for an additional 2 h, a high level of class I transcription was present (Fig. 4D). Quantitative scanning of autoradiograms derived from three separate experiments showed that Ara-C plus IFN-γ treatment resulted in a fivefold increase in class I transcription, whereas DRB plus IFN-γ treatment resulted in a 10-fold increase. A similar increase in class I transcription was observed with cordycepin (30 μg/ml; Sigma) plus IFN-γ (data not shown). These findings can be explained by the notion that partial inhibition of RNA synthesis preferentially decreases the accumulation of RNA coding for a labile repressor factor (or factors) while allowing the accumulation of RNA coding for the necessary positive factor (or factors). Such a result would be expected if the mRNA coding for the negative regulatory factor has a shorter half-life than the mRNA coding for any necessary positive regulatory factor. In agreement with the idea of a labile transcriptional repressor, the run-on transcription assay performed with nuclei from cells treated for 2 h with DRB plus IFN-γ, washed, and subsequently treated with IFN-γ alone for an additional 22 h showed that the level of class I transcription returned nearly to baseline (Fig. 4E), thus indicating that by 24 h, the putative negative factor (or factors) had sufficient time to reaccumulate.

Direct evidence that all of the agents used to stimulate class I transcription in fact inhibit the synthesis of mRNA in K562 cells was obtained in the following experiment. K562 cells were treated first with 0.04 μg of actinomycin D (Sigma) per ml to block subsequent mRNA elongation (29). After 30 min, either DRB (90 μM), Ara-C (1 μM), or 5azaCR (2 μM) was added to the cultures for 4 h. During the final 2 h of incubation, 0.5 mCi of [^{3}H]uridine (20 μCi/μl; Amersham Corp.) was added to the cultures. Total RNA and poly(A)$^+$ mRNA were isolated as described (4). The percent incorporation of [^{3}H]uridine into both total RNA and poly(A)$^+$ mRNA was calculated by counting in a model LS-100 liquid scintillation counter (Beckman Instruments, Inc.). Values were corrected for each sample by normalizing for equal amounts of poly(A)$^+$ mRNA or total RNA. DRB, 5azaCR, and Ara-C inhibited incorporation of [^{3}H]uridine into K562 poly(A)$^+$ mRNA by about 75, 50, and 30%, respectively. Interestingly, a comparison of these data to the transcription data in Fig. 2 and 4 indicates a direct relationship between the extent of inhibition of RNA synthesis and the amount of class I gene transcription that is induced by each agent.

The transcriptional regulation of HLA class I gene expression in the K562 tumor cell model can be explained as follows: a negative factor (repressor), or factors, encoded by a short-half-life mRNA suppresses class I transcription, whereas a positive protein factor, or factors, encoded by a more stable mRNA increases class I transcription but only if the repressor is relatively depleted. Any agent that partially inhibits RNA synthesis or processing favors the accumulation of the positive regulatory factor in the presence of IFN-γ, thus allowing increased HLA class I transcription. This effect is seen with partial inhibition of RNA synthesis by four such agents: 5azaCR, Ara-C, DRB, and cordycepin. Stimulation of class I transcription by IFN-γ requires synthesis of an intermediary early response protein that is inducible by IFN-γ, demonstrated by the observation that inhibition of protein synthesis with CHX completely blocks class I transcription induction by 5azaCR plus IFN-γ, or DRB plus IFN-γ (data not shown).

Beyond the specific case of K562 leukemia cells, this regulatory scheme could account for the decreased class I gene expression in other human tumor cells (8, 18, 31), as well as in undifferentiated embryonic tissues (23). It should also be noted that the transcription data presented here, in conjunction with previous studies showing the ability of IFN-γ alone to increase class I mRNA levels (4), demonstrate that posttranscriptional control mechanisms for HLA class I gene expression are likely to be of major importance in vivo.

In summary, both positive and negative regulatory factors are involved in transcriptional regulation of major histocompatibility complex class I genes in K562 leukemia cells. These results are consistent with evidence of specific viral gene products that can suppress class I expression in both murine and human cells (32) and reports showing the presence of such a repressor in murine thymoma and embryonic carcinoma cells (2, 23). In our studies, we show a repressor effect in a naturally transformed human tumor cell and directly document the site of action to be transcriptional. Our results also document a novel mechanism by which 5azaCR, or any agent that inhibits RNA synthesis, can either directly activate or permit activation of repressed genes without affecting DNA methylation. This latter finding should be taken into account in the design and interpretation of experiments in which 5azaCR is used to alter cellular DNA methylation.

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