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Immunology

Adenovirus type 12 early region 1A proteins repress class I HLA expression in transformed human cells

(major histocompatibility complex/HLA mRNA hybridization/monoclonal antibodies/BK virus plasmid/human interferon γ)

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ABSTRACT **The adenovirus type 12 (Ad12) early region 1A (*E1A*) gene is thought to play a major role in repressing class I major histocompatibility complex expression in transformed rodent cells. However, since transformation by adenovirus requires both *E1A* and *E1B* genes, it has not been demonstrated whether the Ad12 *E1A* gene acts alone or synergistically with the *E1B* gene to accomplish this effect. Moreover, it is not known whether the repression of class I antigen synthesis by Ad12-transforming gene products occurs only in rodent cells. We show that the Ad12 *E1A* gene, in the absence of the *E1B* gene, is capable of greatly reducing the levels of class I HLA antigens and mRNAs in primary human cells transformed by the *E1A* gene of Ad12 and the large tumor antigen (T-antigen) gene of BK virus; control cells transformed by BK virus T-antigen gene alone or the highly related simian virus 40 T-antigen gene showed no apparent alteration in class I HLA expression. Human recombinant interferon γ was able to restore synthesis of class I HLA antigens in transformed cells that produced Ad12 E1A proteins, indicating that these cells were not deficient for class I genes. These results strongly indicate that the Ad12 E1A proteins modulate class I gene expression by similar mechanisms in both transformed rodent and human cells.**

Proteins encoded by the early region 1A (*E1A*) gene of adenovirus both activate and repress expression of other genes. The two E1A proteins of adenovirus type 5 (Ad5) (243 and 289 amino acids) are encoded by overlapping transcripts and are identical except for an internal stretch of 46 amino acids unique to the larger protein (1). Upon infection of human cells by Ad5, the larger of the two E1A proteins facilitates transcription from other early viral promoters (2, 3). The E1A proteins are also known to stimulate transcription of certain endogenous and exogenous cellular genes (4–8). In contrast, E1A proteins repress transcription of other promoters including the E2 late promoter of Ad5 (9) and the simian virus 40 (SV40) early promoter (10, 11).

It is intriguing that in adenovirus-transformed rodent cells, the *E1A* gene of Ad12 plays a central role in the reduction of class I major histocompatibility complex (MHC) antigens and mRNAs, whereas the *E1A* gene of Ad5 exerts no apparent effect (12–14). While both *E1A* and *E1B* genes of Ad5 and Ad12 are sufficient to transform cells *in vitro*, only Ad12-transformed cells are tumorigenic in syngeneic hosts (15–17). Since class I MHC antigens are cell-surface glycoproteins that serve as the key recognition elements for virus-restricted and allospecific cytotoxic T lymphocytes, it has been suggested that lack of class I MHC antigens on the surface of Ad12-transformed rodent cells could help them to escape recognition by cytotoxic T lymphocytes (12–14, 18). The class I antigens are encoded as a multigene family on chromosome 17 of mouse and chromosome 6 of human. It was recently shown that in Ad12-transformed mouse cells, there is diminished expression of antigens and mRNAs from each major class I gene—i.e., H-2K, -D, and -L (13). The Ad12-transforming proteins did not affect transcription of β_2-microglobulin (β_2m), which noncovalently associates with the class I antigens on the cell surface (K.B.E. and R.P.R., unpublished data).

Although the Ad12 E1A protein products are thought to play a major role in repressing class I MHC expression (12), it is not known whether the *E1A* gene acts alone or synergistically with the *E1B* gene to establish this effect in transformed cells. Moreover, it is not known whether the Ad12-transforming gene products will reduce class I MHC expression in species other than rodents. In this study, we show that the Ad12 *E1A* gene, in the absence of the *E1B* gene, is capable of greatly reducing the levels of class I antigens and mRNAs (HLA-A, -B, and -C) in transformed human cells.

MATERIALS AND METHODS

Cell Lines. Primary human embryonic kidney (HEK) cells (Flow Laboratories) were transformed with pBKE1A-12 plasmid DNA to obtain the cell line HBK-E1A-12, and with the *Hha* I–*Eco*RI [3751 base pair (bp)] fragment of BK viral DNA encoding BK large tumor antigen (T antigen) to obtain the cell line HBK. DNA was introduced into cells by the calcium phosphate transfection procedure (19) using 10 μg of DNA per 10^7 cells. SV-1 cells are HEK cells transformed by SV40 (20). The HBK-E1A-12 and SV-1 cell lines were maintained on minimal essential medium supplemented with 8% fetal calf serum (GIBCO), and the HBK cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum.

Reagents. A rabbit antiserum, 12-1A-FP, directed against Ad12 E1A proteins, was used (21). Monoclonal antibodies used were KE2, anti-HLA (22); L368, anti-human β_2m (23); and P3X63Ag8, a nonspecific control antibody (24).

DNA Constructs. The pBKE1A-12 plasmid was constructed by cloning the *E1A* gene of Ad12 into the *Bam*HI site of the vector pMLBK (25). The *E1A* gene was derived by digesting the cloned *Eco*RI C fragment of Ad12 [0–16.5 map units (m.u.); see ref. 21] with the restriction endonuclease *Acc* I to generate a 1599-bp fragment (0–4.57 m.u.), which

Abbreviations: E1A and E1B, early regions 1A and 1B; Ad5 and Ad12, adenovirus types 5 and 12; SV40, simian virus 40; MHC, major histocompatibility complex; β_2m, β_2-microglobulin; bp, base pair(s); BKV, BK virus; IFN-γ, interferon γ; rIFN-γ, recombinant IFN-γ; T antigen, large tumor antigen; m.u., map unit(s).

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was purified from an agarose gel (26). *Bam*HI linkers (New England Biolabs) were added to the 1599-bp fragment, which was then cloned into the *Bam*HI site of pMLBK by standard protocols. The *Hha* I–*Eco*RI (3751 bp) fragment encoding T antigen of the Gardner strain of BK virus (BKV) (27) was purified from an agarose gel (26).

Immunofluorescence. Cell monolayers were acetone-fixed on coverslips and incubated first with a hamster BK T-antigen antiserum followed by a fluorescein-conjugated anti-hamster serum (28).

Cell Sorting. Cell surface antigens were analyzed as described (13) using the monoclonal antibodies KE2 (22) and P3X63Ag8 (24) and a fluorescein-conjugated rabbit anti-mouse IgG (Cappel Laboratories, Cochranville, PA). Cells were analyzed for fluorescence intensity in an Orthofluorograf 50HH.

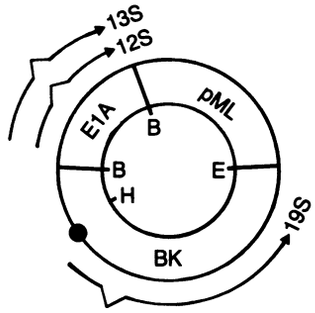
Immunoprecipitations. Cells were metabolically labeled with either $^{32}P_i$ (25 μCi/ml for 5 hr; 1 Ci = 37 GBq) or [^{35}S]methionine (40 μCi/ml for 5 hr), and the membrane fractions were immunoprecipitated with either the 12-1A-FP antiserum as described (21) or monoclonal antibodies using *Staphylococcus aureus* coated with goat anti-mouse antiserum (29). Immunoprecipitates were electrophoresed through $NaDodSO_4$/polyacrylamide gels (30) and fluorographed (31).

RNA Analysis. Poly(A)$^+$ RNA was prepared from total cytoplasmic RNA by chromatography on oligo(dT) cellulose (type 7; P-L Biochemicals). Poly(A)$^+$ RNA (3–5 μg) was denatured and run on a 1% formaldehyde/agarose gel (32), blotted onto nitrocellulose (33), and hybridized with nick-translated DNA probes: HLA-B7 (34), actin pHa4-1 (35), Ad12 E1A (0–4.5 m.u.). The same filter was sequentially hybridized with the different ^{32}P-labeled nick-translated DNA probes ($\approx 1 \times 10^8$ cpm) at 42°C for 22 hr (13) and then washed at a final stringency of 0.1× SSC (1× SSC = 0.15 M NaCl/0.015 M Na citrate) and 0.1% $NaDodSO_4$ at 52°C prior to film exposure at −70°C with an intensifying screen. Removal of specifically hybridized ^{32}P-labeled probe DNA between hybridizations was done by washing in water at 100°C. Densitometric scanning of the RNA bands corresponding to HLA and actin was accomplished by using a Corning 750 densitometer.

RESULTS

Construction and Characterization of Transformed Human Cell Lines. We wished to determine whether the Ad12 *E1A* gene, in the absence of the adenovirus *E1B* gene, was capable of reducing class I MHC expression in transformed human cells. However, complete transformation of primary cells by adenovirus requires both *E1A* and *E1B* genes (15–17). To introduce *E1A* as the only gene of Ad12 into primary human cells and still obtain stable cellular transformants, we co-introduced the T-antigen gene of BK papovavirus, which has the ability to transform primary cultures of human cells (28). This was accomplished by using the plasmid pBKE1A-12 (Fig. 1), in which the *E1A* gene of Ad12 (0–4.57 m.u. of Ad12 DNA) had been cloned into the vector pBK, which contains the complete T-antigen gene of BKV (25). The pBKE1A-12 recombinant plasmid was introduced into HEK cells by calcium phosphate DNA-mediated transfection (19) and foci were grown into a mass culture referred to as HBK-E1A-12; subclones of the HBK-E1A-12 cell line were also established. A control cell line, designated HBK, contained BK T antigen as the only viral gene and was constructed by transfecting the HEK cells with a 3751-bp fragment of BK viral DNA, represented as the *Hha* I–*Eco*RI portion of pBKE1A-12 in Fig. 1. Both the HBK-E1A-12 and HBK cell lines have been in continuous culture for several months.

Expression of viral antigens in the HBK-E1A-12 and HBK cell lines was examined. As shown in Fig. 2*A*, both cell lines synthesized BKV T antigen as detected by positive nuclear



pBKE1A-12

FIG. 1. Structure of the pBKE1A-12 plasmid. The pBKE1A-12 plasmid contains the *E1A* gene of Ad12 (0–4.57 m.u., 1599 bp), the *Eco*RI–*Bam*HI fragment of the plasmid pML (2617 bp), and the *Eco*RI–*Bam*HI fragment of BK viral DNA (5089 bp). The two overlapping *E1A* transcripts of 12S and 13S encode proteins of 235 and 266 amino acids, respectively. The BK DNA sequences include the viral origin of replication (●) and the entire early region encoding the BK large T-antigen 19S mRNA. All transcripts are designated by thin lines with arrows representing the 3′ ends. Restriction endonuclease sites *Bam*HI, *Eco*RI, and *Hha* I are designated B, E, and H, respectively.

fluorescence staining with a polyclonal hamster antiserum to BK T antigen (28). BKV T antigen was also immunoprecipitated from both of these cell lines (not shown). Expression of E1A proteins was analyzed by immunoprecipitation using an Ad12 E1A monospecific antiserum (21). As seen in Fig. 2*B* (lanes 2A–5A), the two major E1A proteins of 39 and 36 kDa (266 and 235 amino acids, respectively) (38, 39) were immunoprecipitated from metabolically labeled cellular extracts derived from both the HBK-E1A-12 mass culture as well as subclones of HBK-E1A-12. Furthermore, the HBK-E1A-12 cells were able to support replication of the E1A-defective mutant viruses, dl312 (40) and hr3, hr4, and hr5 (41, 42) (not shown). As expected, E1A proteins were not immunoprecipitated from extracts of the HBK cell line that had been transfected with only BK DNA (Fig. 2*B*, lane 1A). Thus, both the HBK-E1A-12 and HBK cells produced BK T antigen, whereas only the HBK-E1A-12 cells synthesized the two E1A proteins.

Synthesis of Class I HLA Antigens. Expression of class I HLA antigens by the HBK-E1A-12 and HBK cell lines was examined by flow cytometry and immunoprecipitation using an HLA monoclonal antibody, KE2. The KE2 antibody recognizes a nonpolymorphic determinant, which is common to the 44 kDa protein encoded by class I *HLA-A*, *-B*, and *-C* genes, respectively (22). When the HBK-E1A-12 mass culture and 15 subclones were analyzed for cell-surface expression of class I antigens by flow cytometry using the KE2 antibody, it was found that the percentage of cells expressing class I antigens was reduced by a factor of 3–7 compared to that of control cell lines, which included the HBK cells, nontransformed HEK primary cells, and an SV40-transformed HEK cell line, SV-1 (20). It is noted that the SV-1 cells were used as an additional control, since the SV40 T-antigen-transforming protein has 74% amino acid sequence homology to BKV T antigen and is functionally similar (43, 44). This reduced expression of class I HLA antigens on the surface of HBK-E1A-12 cells could be due to lowered synthesis of HLA proteins. The KE2 antibody was used to evaluate the presence of class I HLA molecules from the membrane fraction of labeled cell extracts. As shown in Fig. 3, the 44-kDa class I HLA proteins could be readily immunoprecipitated from extracts of HBK cells (lane 1B) and SV-1 cells (lane 3B). In marked contrast, class I HLA proteins were weakly immunoprecipitated from the HBK-E1A-12 mass culture (lanes 2B and 6B) and subcloned cell lines (lanes 4B and 5B). The 12-kDa β_2m protein, which noncovalently associates with HLA antigens on the cell-surface membrane, was detected

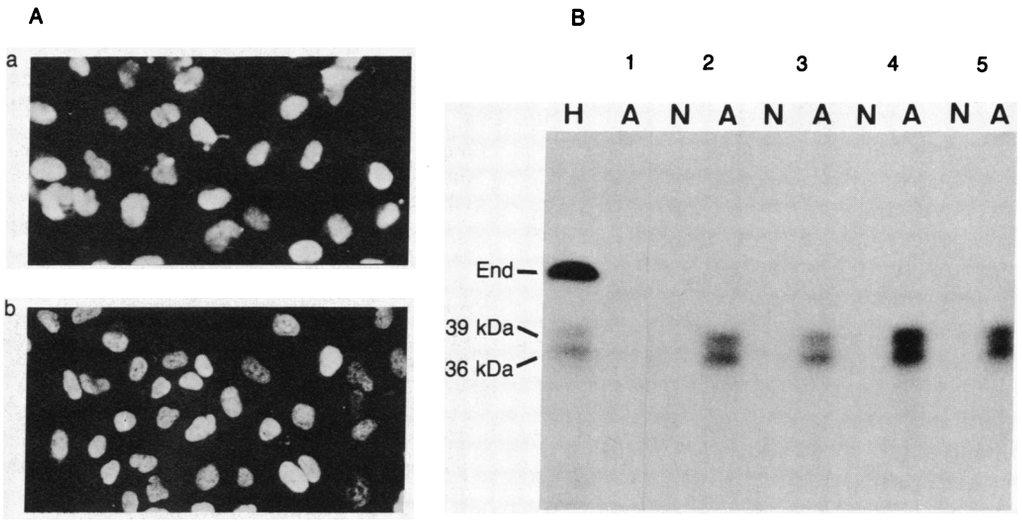


FIG. 2. Expression of BK T antigen and Ad12 E1A proteins in transformed human embryonic kidney cells. (*A*) Indirect immunofluorescence of BKV T antigen in HBK (*a*) and HBK-E1A-12 (*b*) transformed cells. Both the HBK and HBK-E1A-12 cells show positive nuclear fluorescence. (*B*) Immunoprecipitation of Ad12 E1A proteins from HBK-E1A-12 cells. ^{32}P-labeled extracts of HBK and HBK-E1A-12 cells were immunoprecipitated, electrophoresed through 10% $NaDodSO_4$/polyacrylamide gels, and fluorographed; 10^6 trichloroacetic acid-precipitated cpm of the membrane-containing fraction was used in each immunoprecipitation. Immunoprecipitates using the Ad12 E1A monospecific rabbit antiserum, 12-1A-FP, are marked (A) and those using the normal (control) rabbit antiserum are marked (N). Lane H contains Ad12 E1A proteins of 39 and 36 kDa (266 and 235 amino acids, respectively), which were translated *in vitro* from RNAs obtained from an Ad12-transformed hamster cell line, HA12-7 (36), that had been hybridization-selected (37) to a fragment encoding the Ad12 *E1A* gene; End, endogenously labeled protein. Cell lines used were HBK (lane 1), HBK-E1A-12 mass culture (lanes 2), HBK-E1A-12 subclones 2, 5, and 8 (lanes 3, 4, and 5, respectively).

in all cell lines either by coprecipitation with HLA using the KE2 monoclonal antibody (lanes 1B and 3B) or by direct immunoprecipitation with a β_2m monoclonal antibody (23) (lanes 3C–6C).

Steady-State Levels of Class I mRNAs. The reduced levels of class I HLA antigens in the HBK-E1A-12 cells could result from a decrease in the level of class I HLA mRNAs. Polyadenylylated RNA from HBK-E1A-12, HBK, and SV-1 cells was size-fractionated on formaldehyde/agarose gels, transferred to nitrocellulose paper, and analyzed with an HLA cDNA probe (HLA-B7) that cross-reacts with all class I HLA mRNAs (34). As seen in Fig. 4, the steady-state level of class I HLA mRNAs in both the HBK-E1A-12 mass culture (lanes 2 and 5) and subclone 8 (lane 4) was greatly

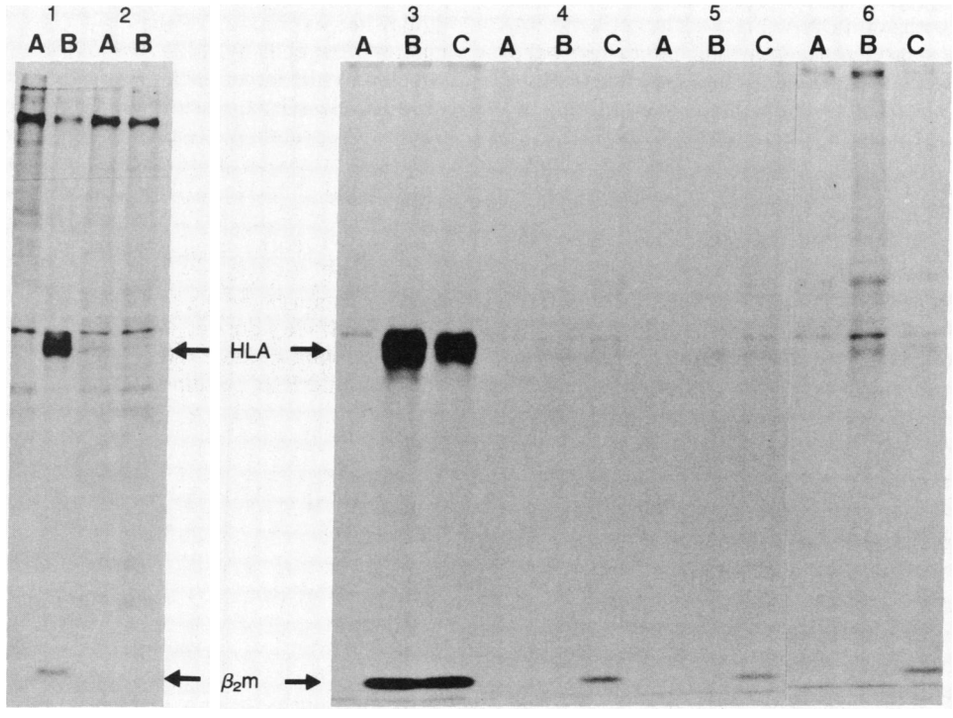


FIG. 3. Immunoprecipitation of class I HLA antigens and β_2m from transformed HEK cells. [^{35}S]Methionine-labeled cell extracts were immunoprecipitated with different monoclonal antibodies, electrophoresed through 15% $NaDodSO_4$/polyacrylamide gels, and fluorographed. Each lane represents immunoprecipitation of 2×10^7 trichloroacetic acid-precipitated cpm of membrane extract. Monoclonal antibodies used were P3X63 Ag8 (control), lanes A; KE2 (anti-HLA), lanes B; L368 (anti-β_2m), lanes C. Cell lines used were HBK (lanes 1); HBK-E1A-12 mass culture (lanes 2 and 6); HBK-E1A-12 subclones 8 and 6 (lanes 4 and 5, respectively); and SV-1 cells (lanes 3).

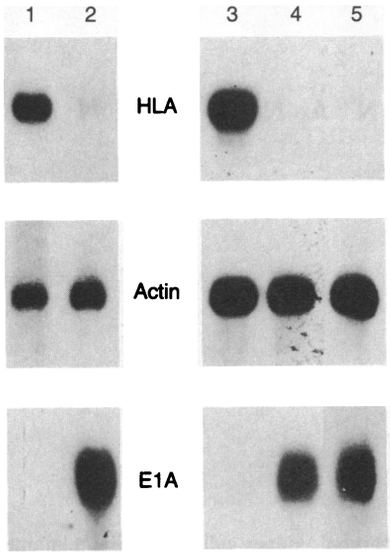


FIG. 4. Levels of class I HLA mRNA in transformed HEK cells. Poly(A)$^+$ RNAs from HBK cells, HBK-E1A-12 cells, and SV-1 cells were electrophoresed on a 1% formaldehyde/agarose gel, transferred to nitrocellulose paper, and probed with ^{32}P-labeled nick-translated DNA. The same filter was sequentially probed with an HLA cDNA molecule (HLA-B7) that cross-hybridizes with each class I HLA transcript—i.e., HLA-A, -B, -C (34), actin DNA (pHa4-1), and Ad12 E1A (0–4.5 m.u.) DNA. Cell lines represented are HBK (lane 1), HBK-E1A-12 mass culture (lanes 2 and 5), HBK-E1A-12 subclone 8 (lane 4), and SV-1 (lane 3).

reduced in comparison to the levels of these mRNAs in the HBK and SV-1 control cell lines (lanes 1 and 3, respectively). The diminished amounts of class I HLA mRNAs in the HBK-E1A-12 cells was not due to degradation of cellular RNA, since comparable levels of actin mRNA were present in the HBK-E1A-12, HBK, and SV-1 cell lines when the same blot was reprobed with actin DNA (Fig. 4). The actin mRNA was used as an internal reference with which to compare the relative amounts of class I HLA mRNA since adenovirus infection has been shown not to affect actin transcription (5, 45). Densitometric scanning of the autoradiogram in Fig. 4 revealed that the ratio of HLA to actin mRNA was reduced 97% in the HBK-E1A-12 mass culture as compared to the HBK cell line. When the same blots were reprobed with Ad12 E1A, β_2m, and BK (or SV40) T-antigen DNAs, respectively, the E1A mRNAs were detected only in the HBK-E1A-12 cells (Fig. 4), whereas the β_2m and T-antigen mRNAs were readily observed in all the cell lines (not shown).

Effect of Interferon γ (IFN-γ) on Class I Antigen Expression. IFN-γ is known to stimulate cell-surface expression of MHC antigens of fibroblasts (46). Moreover, IFN-γ was shown to restore synthesis of each class I H-2 antigen (H-2K, -D, and -L) on the surface of Ad12-transformed mouse cells (13). Reduction of class I HLA antigens in the HBK-E1A-12 cells could not be attributed to a defect in the class I genes since human recombinant IFN-γ (rIFN-γ) dramatically increased the levels of class I antigens on the cell surface. For example, when the HBK-E1A-12 subclone 8 cell line was treated with rIFN-γ (1000 units/ml for 48 hr), the percentage of cells that reacted with the KE2 class I HLA monoclonal antibody increased from 4% (nontreated) to 60% (rIFN-γ treated) as measured by flow cytometry as shown in Fig. 5. These same cells showed a marked increase in the levels of class I transcripts after treatment with rIFN-γ (not shown).

DISCUSSION

These results demonstrate that the Ad12 *E1A* gene is able to greatly reduce class I MHC expression in the absence of the *E1B* gene in transformed cells. Furthermore, these findings reveal that the diminished expression of class I MHC genes by Ad12 E1A is a phenomenon that is not restricted to rodent cells (12–14) but now can be extended to human cells. Since the HBK-E1A-12 cells in this study synthesize both Ad12 E1A and BK T-antigen proteins, we cannot eliminate the possibility that BK T antigen may act synergistically with the E1A proteins to reduce class I gene expression. However, HEK cells transformed exclusively by the BK T-antigen gene (HBK cells) or the highly homologous SV40 T-antigen gene (SV-1 cells) exhibited no apparent alteration of class I expression as compared to the nontransformed HEK cells.

Reduced levels of class I HLA antigens and mRNAs in the HBK-E1A-12 transformed cells is correlated with synthesis of the Ad12 E1A proteins. The fact that rIFN-γ was able to activate expression of class I HLA antigens on the cell surface excluded the possibility that these cells were deficient for class I genes. Presumably, one or both of the Ad12 E1A proteins (266 and 235 amino acids) are responsible for diminished expression of the class I *HLA* genes. Transformation of rat cells by Ad12 *E1B* and a mutated *E1A* gene, in which only the larger of the E1A proteins is affected, failed to block class I expression and thereby suggested that the larger E1A protein plays a major role in the shut-off of class I MHC expression (18). Moreover, transformation of rat cells by *E1B* and chimeric Ad12–Ad5 *E1A* genes has implied that the first exon of the Ad12 *E1A* gene modulates expression of class I MHC antigens (47). Surprisingly, infection of mouse embryo cells by Ad5 or Ad12 resulted in an increase in class I transcription, although the amounts of class I antigens on the cell surface were unchanged (48). The activation of class I transcription in these infected cells was attributed to both the E1A and E1B proteins (48).

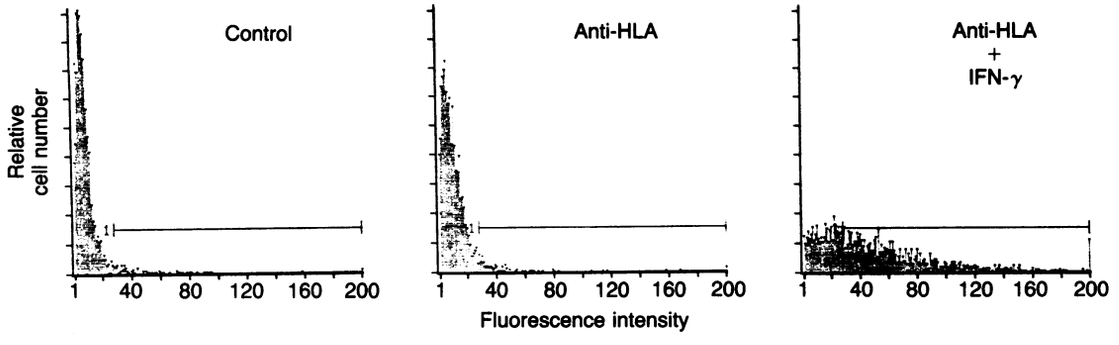


FIG. 5. Effect of IFN-γ on expression of HLA cell-surface antigens of HBK-E1A-12 cells. HBK-E1A-12 cells (subclone 8) were incubated with or without human IFN-γ (1000 units/ml) and were analyzed 48 hr later for expression of cell-surface HLA antigens by flow cytometry using the KE2 monoclonal antibody.

Reduced class I gene expression by Ad12 has been reported only for primary cultures that have been the recipients of the viral transforming region (12–14). In preliminary experiments, we co-introduced the cloned Ad12 *E1A* and *E1B* and BK T-antigen genes into an established TK^- human osteosarcoma cell line, 143B (49). Although TK^+ cellular transformants containing these genes synthesized detectable levels of E1A proteins, there was no apparent decrease in expression of class I HLA antigens. This suggests that reduction of class I antigen expression by the Ad12 *E1A* gene requires either a critical cellular concentration of E1A proteins or the direct involvement of E1A proteins in the transformation process or a specific cell type.

Recently, it was shown that expression of MHC antigens from each major class I locus (*H-2K*, *-D*, and *-L*) was reduced in Ad12-transformed mouse cells (13). Thus, it is also likely that in the HBK-E1A-12-transformed human cells, the Ad12 E1A protein products affect expression of MHC antigens that originate from each class I locus (*HLA-A*, *-B*, *-C*). Support for this stems from the fact that both the class I HLA monoclonal antibody and the cDNA probe used in this study recognize sequences common to each class I antigen and mRNA, respectively. Taken together, these results strongly indicate that the Ad12 E1A proteins repress class I gene expression by similar mechanisms in both transformed rodent and human cells. In this regard, it is significant that in both mouse (13) and human transformed cells, stimulation of transcription of class I genes by IFN-γ is dominant over the repressive effect of the Ad12 E1A proteins. Recent evidence suggests the existence of a *trans*-acting regulatory gene for HLA class II genes that is unlinked to the MHC (50). It is intriguing to consider that the *E1A* gene products could indirectly regulate each of the class I genes by altering expression of an analogous class I regulatory gene.

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