

Interferon System Defects in Human Malignant Melanoma

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ABSTRACT

We have examined the ability of melanoma cell lines and normal human melanocytes, which have demonstrable intact IFN genes, to secrete both IFN- α and IFN- β in response to induction with virus. Normal melanocytes were found to secrete both IFN- α and IFN- β after virus induction. In contrast, although all but one of the melanoma lines tested were capable of secreting IFN- β , none were capable of IFN- α secretion. This phenomenon was not due to defects in either translation of IFN- α mRNA or secretion of IFN- α proteins, since transfection of melanoma lines with a constitutive IFN- α 2b expression vector resulted in the secretion of high levels of IFN. On further examination, this inability to express natural IFN- α appeared to be due to a defect in activation of the IFN- α promoters, since constructs containing the IFN- α promoter were completely unresponsive to viral infection in melanoma cells but inducible in melanocytes. These results show that there is a specific disruption of IFN- α gene activation rather than IFN- β in melanoma lines and suggest that this is due to disruption of a trans-acting IFN- α gene transcription factor. Disruption of this factor and its consequences may be important in the development of malignant melanoma.

INTRODUCTION

Malignant melanoma is a particularly aggressive tumor that often proves refractory to conventional chemotherapy. The most effective biological therapeutic agent tested thus far is IFN- α (1). Clinical trials have evaluated IFN- α as a single-agent treatment in patients with metastatic melanoma (reviewed in Refs. 1 and 2). Although the response rates vary (11–38%, including both partial and complete remissions), these studies universally report therapeutic benefit of IFN- α in a proportion of melanoma patients. In addition, high local tissue concentrations of IFN- α after intralesional application into metastatic lesions led to regression of >50% of the metastases (3, 4). These results support the view that IFN- α has clinical value in the treatment of metastatic melanomas. Whether, as seen with chronic myelogenous leukemia (5), a far greater benefit would be observed on IFN- α treatment of melanoma tumors at a less advanced stage in their development is unknown.

Cytogenetic analysis of both sporadic and familial melanoma report frequent loss or rearrangement within the short arm of chromosome 9, specifically bands 9p13–p22 (6–13). These rearrangements occur at early stages of melanoma development (14, 15). Analysis of malignant melanoma lines has provided evidence for a potential tumor suppressor and, therefore, candidate susceptibility gene *CDKN-2* (p16) within this locus (16, 17), although contradictory reports regarding the frequency of *CDKN-2* mutation or loss in familial melanoma and freshly isolated sporadic melanomas have subsequently been made (18–20). The IFN- α family of genes and the single IFN- β gene have also been located within this region at 9p21–22. Because these cytokines have been shown to exhibit diverse biological properties that may act against tumor cell survival (reviewed in Ref. 21), they are also potential candidates as tumor suppressors. Genetic mapping of 9p21–22 has defined the shortest region of overlap of the

9p deletions observed in melanoma to 40 kb within chromosome band 9p21, with homozygous deletions occurring in >55% (46 of 84) of melanoma lines tested (22). This region excludes the type I IFN genes that are located in band 9p22 (23, 24), with the 23 members of the IFN- α gene family occurring within a single gene locus spanning over 230 kb just proximal to the IFN- β 1 locus (25). Nevertheless, the critical 9p21 region of deletion lies in close proximity and just centrosomal to the IFN- α gene cluster (22). It is feasible that this region may contain a *cis*-acting dominant control region that regulates transcription from the multigene IFN locus in a similar manner to that observed for regulation of the β -globin gene family (26). It is, therefore, of interest to examine whether melanoma cells that have 9p21 rearrangements and yet contain intact IFN genes are capable of the regulated expression of these genes.

Although there have been a number of studies published on the effects of IFNs on the proliferation and differentiation of melanoma cells (27, 28), to date little has been done to examine the ability of melanocytic cells to express and secrete IFNs (29, 30). Studies examining human melanocytes and melanoma cell lines under normal culture conditions (30) have revealed mRNA from the IFN- β gene by reverse transcription-PCR in 17 of 21 melanoma lines tested. No mRNA for either IFN- α or IFN- γ was detected. Transcription from the IFN- α , IFN- β , and IFN- γ genes was not detected in normal melanocytes. *In vitro*, expression of IFNs by cells under normal growth conditions is generally rarely detected but can be induced by viruses or double-stranded RNA (31). IFN- α mRNA has, however, been detected in normal tissues *in vivo*, including the basal layer of the epidermis, and may contribute towards the normal control of cellular proliferation and differentiation (32, 33). Although IFN expression *in vivo* can occur due to virus infection, the existence of nonviral physiological stimuli is also suspected (31).

We have, therefore, examined the ability of seven melanoma cell lines together with normal human melanocytes, both with and without induction by SV, to secrete IFN- α and IFN- β . None of the cells tested were found to secrete IFN- α or IFN- β constitutively, whereas all but one were capable of IFN- β secretion after optimal induction with virus. In contrast, only normal melanocytes were found to secrete IFN- α after virus induction. Our findings conclude that normal regulation of the expression of IFN- α genes is disrupted in these melanoma cells, whereas that of IFN- β is not. In addition, we found that this disruption appears to occur at the promoter level, with promoter activation being defective. This defect does not appear to occur due to the loss or mutation of the IFN- α promoters, nor to a defect in a *cis*-acting dominant control region. We, therefore, hypothesize that a *trans*-acting gene that specifically regulates the activity of the IFN- α gene promoters and not the IFN- β promoter is disrupted in these cells and that this gene may be important in the pathogenesis of malignant melanoma.

MATERIALS AND METHODS

Cell Culture of Melanoma Cell Lines. All lines used originate from metastatic cutaneous melanoma: A375-P (34), DX-3 (35), SK28 (36), SK23 (37), and HMB-2 (38). In addition, two cell lines, A375-M (34) and LT-5 (35), were used that were more metastatic variants of A375-P and DX-3, respectively. Cells were cultured in DMEM (GIBCO-BRL), 10% FCS² (Advanced

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Protein Products), 100 units/ml penicillin, 100 µg/ml streptomycin, and 4 mM L-glutamine (GIBCO-BRL).

Cell Culture of Normal Human Melanocytes. Strains of melanocytes were initiated from neonatal foreskins. Briefly, the epidermis/dermis was trimmed of loose connective tissue, finely minced, and trypsinized for 1–2 h at 37°C. The resulting cell suspension was plated into melanocyte growth medium, which was made up of MCDB153 medium (Sigma Chemical Co.) containing the following: 4×10^{-5} mM CaCl_2 , 10^{-4} M 3-isobutyl-1-methylxanthine (Sigma), 10^{-9} M cholera enterotoxin (ICN), 5 µg/ml bovine insulin (Sigma), 5 µg/ml human transferrin (Sigma), 0.2 µg/ml hydrocortisone (Sigma), 5 µg/ml human recombinant basic fibroblast growth factor (Sigma), 6.1×10^{-4} % (v/v) ethanalamine (Sigma), 14 µg/ml phosphoethanolamine (Sigma), and 3% chelated FCS (Advanced Protein Products). The media was replaced twice/week, and cultures were split 1:3 on confluency. Only pure melanocyte strains (at passage 3–5) were used for experiments. Cellular contamination of cultures with keratinocytes or fibroblasts was assessed using a panel of specific cell-type markers.

DNA Preparation and PCR Amplification. Genomic DNA samples were prepared in the following manner. Cells (10^6) were resuspended in 100 µl buffer A (75 mM NaCl and 25 mM EDTA, pH 8). Cells were then lysed by the addition of 100 µl buffer B [10 mM Tris (pH 8), 10 mM EDTA, 1% SDS, and 400 µg/ml proteinase K] and incubation at 50°C for 3 h. This preparation was then gently extracted with phenol, 1:1 phenol/chloroform, and chloroform followed by ethanol precipitation. The PCR primers used for the amplification of IFN- α genes were those described previously² for the cloning of IFN- α 2 genes from normal lymphocyte cDNA, and since IFN- α genes are intronless, they can be used for amplification of the genes from genomic DNA. These primers are: BK40, ACACGAAGCTTATAACCACCACCATGGCCTTGACCTTGGCTTACTGG (which includes the first 25 bases of the translated sequence of IFN- α 2); and BK41, GTTAGAATTCTAGATCATTCTACTTACTTCTAAACTTCTTGC (which includes sequence complementary to 28bp of the 3' translated sequence prior to and including the termination codon of IFN- α 2). Underlined sequences represent extensions of the primers to incorporate HindIII (BK40) and EcoRI (BK41) restriction sites for subcloning. Although these primers were originally designed to specifically amplify IFN- α 2 sequences, due to close homology of the IFN- α genes, they also amplify IFN- α 1, IFN- α 13, and IFN- α 5.² PCR amplifications were performed using the Hybaid thermal cycler with the following program: thirty cycles of denaturing at 94°C for 1 min, annealing at 50°C for 2 min, and elongation at 72°C for 3 min. Taq polymerase was supplied by Perkin-Elmer.

Interferon Secretion Assay. Trypsinized cells were plated at 2×10^5 cells/35-mm Petri dish in 2 ml of the relevant normal growth medium and allowed to adhere for 48 h. Pretreatments were added directly to the medium at the relevant times before SV treatment. Note: the wells pre-treated with IFN- α were washed five times with normal growth medium before further treatment. SV was added directly to the medium, and the cells were incubated at 37°C for an additional 48 h before the media was harvested. Media was centrifuged to remove cell debris and frozen until use. For IFN α ELISA, 96-well ELISA plates were coated overnight at 4°C with 100 µl/well of 2 µg/ml bovine anti-human IFN- α (Wellcome Foundation, Ltd). Wells were washed five times with 0.1% Tween 20 in PBS and blocked with 3% BSA in PBS overnight at 4°C. After washing, 100 µl of samples were added per well and incubated for 1 h at 37°C. IFN- α was detected with a 1:2000 dilution of anti-IFN- α /HRP-conjugated polyclonal antibody (Wellcome Foundation, Ltd). Antibody binding was visualized with Sigma TMB substrate solution. The reaction was read at an absorbance of 450 nm. Minimum detectable concentration of IFN- α using this ELISA is 0.2 ng/ml. For IFN- β ELISA, quantitation of IFN- β in culture media was performed using the specific immunoenzymetric assay IFN- β -EASIA 228 (MEDGENIX Diagnostics SA) in accordance with the supplier's instructions. Minimum detectable concentration of IFN- β using this kit is 0.01 ng/ml.

Plasmids. The *in vitro* transcriptional activation of IFN- α and IFN- β genes requires regulatory DNA sequences, known as VREs,³ located 5' of these

genes. These VREs are inducible by virus and dsRNA (39). The IFN- α promoter construct, $\text{p}\alpha_1\text{tkSAP}$,⁴ contains a 46-nucleotide VRE segment from the IFN- α 1 gene (40), upstream of the HSVTK minimal promoter (41) driving the reporter gene, SAP. The IFN- β promoter construct, $\text{p}\beta\text{tkSAP}$,⁴ contains the VRE of the IFN- β 1 gene, upstream of the HSVTK minimal promoter driving SAP. A plasmid containing the human EF 1 gene promoter (42) driving SAP was used as the control for successful transfection and is designated pEFSAP. pEEIFN- α 2b contains the IFN- α 2b gene driven by the strong CMV-IE promoter⁵ and is a constitutive expression plasmid.

Transfection Procedure. Trypsinized cells were plated at 2×10^4 cells/well in 48-well plates and allowed to adhere for 48 h. The wells were then washed three times with serum-free media and 200 µl serum-free media containing 11 µg/ml Transfectam (Promega), and 2.1 µg/ml of the appropriate plasmid were added per well. Plates were incubated for 1.5 h at 37°C. The medium was replaced with 200 µl of normal growth media, with or without SV, and incubated for 48 h, when the supernatants were harvested for either SAP or IFN- α assays.

SAP Assay. Media samples were incubated at 65°C for 20 min to inactivate any nonplacental alkaline phosphatases present in the media. Serial dilutions of the cooled samples were added (20 µl) to 180 µl of substrate solution [2 mg/ml *p*-nitrophenyl phosphate (Sigma) in 1 M diethanolamine and 0.5 mM MgCl_2 at pH 9.5] in 96-well plates and incubated at 37°C for 2–12 h until the reaction had developed. Plates were read at an absorbance of 405 nm.

Proliferation Assay. Cells were seeded into 48-well plates at 1×10^4 cells/well and allowed to adhere in normal growth media for 48 h. IFN- α 2b (human recombinant; Wellcome Foundation, Ltd.) was titrated between 100 and 10,000 IU/ml in triplicate for each cell line. IFN- α 2b was added in fresh media twice weekly. Since there is a large difference in proliferation rates between the melanoma lines and normal melanocytes, the relative cell numbers were measured after 5 and 14 days growth, respectively. Relative cell numbers were estimated by staining the cells with crystal violet solution (0.5% crystal violet, 0.85% NaCl, 5.0% formalin, and 50% ethanol) for 10 min at room temperature. The cells were then washed five times with PBS and solubilized with 100 µl 33% acetic acid. Samples were read at an absorbance of 540 nm.

RESULTS

To establish whether each of the melanoma lines contained IFN- α genes, genomic DNA was prepared from each line and used as a template for PCR amplification. The primers BK40 and BK41, which anneal to IFN- α 1, 2, 5, and 13 sequences, were used and PCR products were analyzed using agarose gel electrophoresis. A band of the correct size of 570 bp was obtained for all of the cell lines used (Fig. 1). Since this technique is not quantitative, a single allelic deletion or even multiple gene deletion would not be detected from any of these cell lines; however, the presence of the 570-bp band indicates that at least one IFN- α gene is present in all of the cell lines tested.

The melanoma cell lines, along with normal human melanocytes, were examined for their ability to secrete IFN- α and IFN- β , both constitutively and in response to SV. In the absence of induction by virus, none of the melanoma lines or melanocyte strains examined secreted detectable levels of either IFN- α or IFN- β . The concentration of virus required for optimal induction of IFN secretion was titrated in the range of 3,500–120,000 HAU/ml (data not shown). Optimum secretion of both IFN- α and IFN- β , by cells which were capable of doing so, occurred on induction with 15,000 HAU/ml of SV. The level of IFNs secreted by each cell line and a representative strain of normal human melanocytes after induction with this optimal concentration of SV is indicated in Table 1. None of the melanoma cell lines were capable of secreting detectable levels of IFN- α under these conditions, whereas normal melanocytes secreted 26.6 ng IFN- α /10⁶ cells. In

² C. Linge, D. Gewert, J. Ellis, D. Tucker, G. Allen, and J. S. Crowe. Transcription of IFN- α 2 alleles from virus-induced human leukocytes and lymphoblastoid cells of African origin, submitted for publication.

³ The abbreviations used are: VRE, virus response element, dsRNA, double-stranded RNA; SAP, secreted alkaline phosphatase; EF, elongation factor; SV, Sendai virus.

⁴ C. L. Dent, S. J. MacBride, N. A. Sharp, and D. R. Gewert. Differential levels of inducibility of virus response elements of the human IFN α subtype genes, manuscript in preparation.

⁵ C. Rossmann, N. Sharp, and D. Gewert, manuscript in preparation.

1 2 3 4 5 6 7 8 9 10 11



Fig. 1. One % agarose gel showing PCR reactions of genomic DNA samples with IFN- α -specific primers BK40 and BK41. Lanes 1 and 11, 100-bp ladder; Lane 2, A375-P; Lane 3, A375-M; Lane 4, DX-3; Lane 5, HMB-2; Lane 6, LT-5; Lane 7, SK23; Lane 8, SK28; Lane 9, positive control of a lymphoblastoid cell line, Namalwa; Lane 10, normal melanocytes.

Table 1 Assay of SV-induced IFN secretion

	A375-P	A375-M	DX-3	HMB-2	LT-5	SK23	SK28	NM ^a
IFN- α (ng/10 ⁶ cells)	0	0	0	0	0	0	0	26.6
IFN- β (ng/10 ⁶ cells)	>40.0	>40.0	40.0	9.3	>40.0	16.5	0	11.8

^a NM, normal melanocytes; 0, not detectable.

contrast, all cells secreted IFN- β at levels of greater than 9.3 ng/10⁶ cells (Table 1). There was no difference seen in the ability to secrete IFNs between the cell lines DX-3 and A375-P and their metastatic clones LT-5 and A375-M, respectively.

Pretreatment of cells with sodium butyrate (1 mM for 24 h), which is commonly used to potentiate virus-induced IFN secretion (43), increased IFN- α secretion (by a factor of 2) from the normal melanocytes, but melanoma-secreted IFN- α remained undetectable (Table 2). The possibility that the melanoma cells were not infected by SV is ruled out by the fact that most melanoma cell lines were induced to secrete IFN- β under the same conditions (Table 1). The melanoma cell line SK28, however, did not secrete detectable levels of either IFN- α or IFN- β , even after butyrate treatment (Table 1). Nevertheless, SK28 did appear to have been infected by SV, as considerable cell death had occurred 24 h after addition of the virus (data not shown).

To examine whether the IFN- α translation and secretion mechanisms of the melanoma cells were intact, we transfected these cells with the constitutive IFN- α 2b expression plasmid, pE2IFN α 2b. Conditioned media assayed for IFN- α was found to contain high levels (>140 ng/10⁶ cells) of IFN- α from all of the melanoma cell lines (Table 2). The IFN- α translation and secretion mechanisms, therefore, appear to be intact for all of the melanoma cell lines tested in this study.

Since none of the melanoma lines tested appear capable of producing any form of IFN- α upon induction and yet all cell lines are capable of secreting IFN- α 2b when driven by an adequate promoter, this loss of IFN- α secretion ability appears to occur at the level of transcription. It is highly unlikely that the promoter of each IFN- α gene has been lost or mutated in such a way as to make it inactive. It is more conceivable that there is some dysfunction in a general factor that specifically controls the activity of the IFN- α promoters but not the IFN- β promoters. Also, although the genetic status of the cell lines used is unknown, genetic rearrangements of 9p21 are extremely common in melanoma cell lines (23, 22). Since the affected 9p21 region is just centromosomal to the IFN- α gene cluster, this region may contain a *cis*-acting dominant control region that controls the activity of the IFN- α gene family. Disruption of this putative *cis*-activation factor may, therefore, be responsible for loss of IFN- α gene activity in our melanoma cell lines. To test this hypothesis, we transfected normal melanocytes and each melanoma line with a panel of plasmids. For each cell type, six wells were transfected with each of one of the following promoter/reporter-gene plasmids: p α ,tkSAP, p β tkSAP, or pEFSAP. The latter plasmid acts as a control for successful transfection and also for the general level of transcription within the cells. The IFN- β promoter plasmid acts as a control for viral activation of the IFN system. Media collected from untransfected cells were used as SAP -ve controls for each cell line/strain tested. Three wells/plasmid/

Table 2 IFN- α assay after pretreatment and SV induction

Pretreatments	A375-P	A375-M	DX-3	HMB-2	LT-5	SK23	SK28	NM ^a
None	0	0	0	0	0	0	0	28.8
Sodium butyrate	0	0	0	0	0	0	0	56.0
IFN- α ^b	0	0	0	0	53.8	0	0	22.8
pE2IFN α ^c	175.4	298.4	276.3	480.1	304.4	140.7	268.3	ND

^a NM, normal melanocytes; 0, not detectable; ND, not done.

^b Units are ng of IFN- α /10⁶ cells secreted in 48 h.

^c Transient expression level of IFN- α driven by cytomegalovirus promoter.

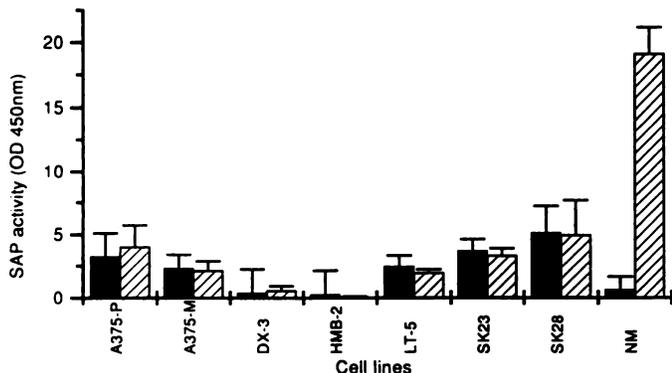


Fig. 2. pα1tkSAP activity expressed as a percentage of positive control plasmid pEFSAP. NM, normal melanocytes; ■, no SV; ▨, plus SV. Bars, SD from the mean of triplicate wells.

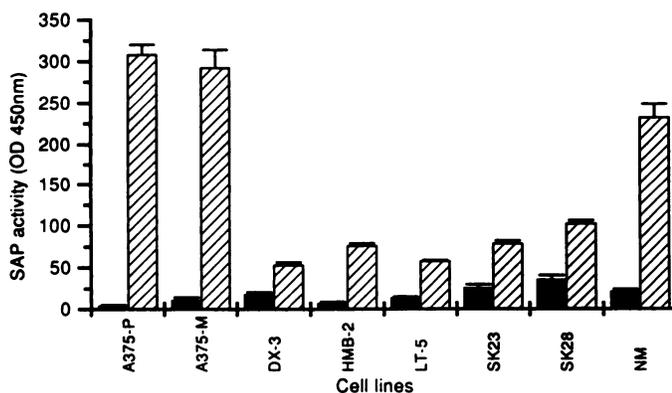


Fig. 3. pβtkSAP activity expressed as a percentage of positive control plasmid pEFSAP. NM, normal melanocytes; ■, no SV; ▨, plus SV. Bars, SD from the mean of triplicate wells.

cell type were then induced with SV, whereas the remaining three were not, and the conditioned media (harvested after 48 h) was assayed for SAP activity (Figs. 2 and 3). In contrast to the normal melanocytes, none of the melanoma cell lines tested showed significant activity of the IFN-α promoter above background (Fig. 2), whereas both normal melanocytes and all of the melanoma lines showed significant activity of the IFN-β promoter (although levels varied; Fig. 3). Interestingly, the melanoma line SK28, which is incapable of secreting IFN-β, showed virus-induced activity of the IFN-β promoter.

Virus induction of IFN-α promoter activation, specifically, is disabled in all melanoma lines tested. These results suggest that a factor that controls the activity of the IFN-α promoters is either missing or mutated in these cells and that this factor acts in *trans*- rather than *cis*-.

It may be that this inability of melanoma cells to produce IFN-α could play an important role during tumor development, and since at least a proportion of melanoma patients (11–38%) respond to systemic therapy with IFN-α alone, it is important to establish the role of IFN-α in the survival of melanoma cells. As it is known that pretreatment of cells with IFN potentiates the subsequent IFN secretion response on SV induction (44), we examined the effects of IFN-α2b pretreatment on IFN-α secretion by melanoma cell lines and melanocytes in response to SV induction. The concentration of IFN-α2b (1,000–13,000 IU/ml) and duration of IFN pretreatment (30 min–24 h) was titrated (data not shown). The optimum response, by cells that were capable of doing so, was found to occur with a 2-h preincubation with 1300 IU/ml of IFN-α2b. The results after optimal conditions of IFN-α pretreatment are shown in Table 2. Sister wells were pretreated

in the same manner but not induced with SV as a negative control. IFN-α pretreatment had no effect on the level of SV-induced IFN-α secretion by normal melanocytes under any conditions. Similarly, the majority of the melanoma lines (6 of 7) remained unable to produce detectable IFN-α after IFN-α pretreatment and SV induction. However, the melanoma cell line LT-5 was optimally induced to secrete a similar level of IFN-α to that produced by normal melanocytes.

These results were mirrored by the effects of IFN-α2b pretreatment on SV induction of the IFN-α promoter plasmid (pα1tkSAP) transfected into LT-5 cells. In the absence of IFN-α pretreatment, the IFN-α promoter appears completely inactive, but after optimal IFN-α pretreatment, activity of the promoter (Fig. 4) reaches similar levels to that seen in normal melanocytes (Fig. 2). These results seem to suggest that, at least for one melanoma line, the inactivity of the IFN-α promoter can be reversed by treatment with IFN-α.

We further examined the effects of IFN-α treatment by determining its antiproliferative action on the melanoma lines and several strains of normal human melanocytes. IFN-α2b was titrated from 100–10,000 IU/ml, and only the minimum and maximum effective levels have been shown in Fig. 5. In agreement with data published previ-

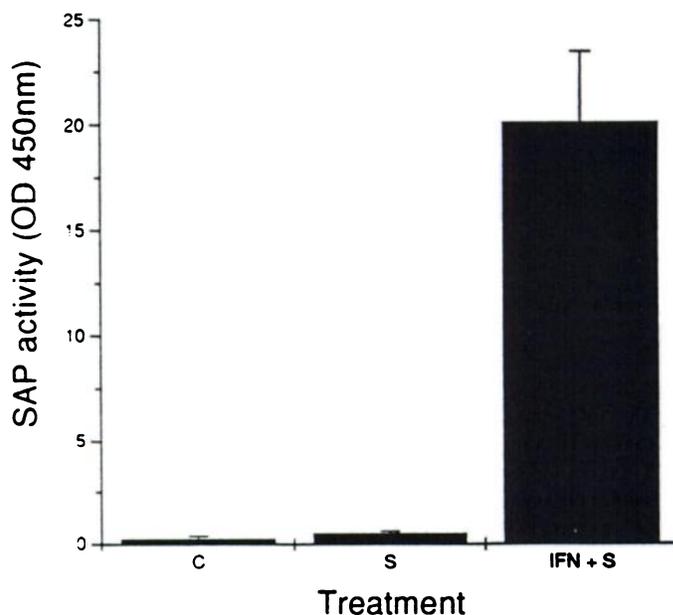


Fig. 4. Effect of IFN-α2b pretreatment of LT-5 cells on IFN-α promoter activity. pα1tkSAP activity is expressed as a percentage of positive control plasmid pEFSAP. C, no pretreatment, no SV; S, no pretreatment, plus SV; IFN + S, pretreatment with 1,300 IU/ml IFN-α2b for 2 h, followed by SV. Bars, SD from the mean of triplicate wells.

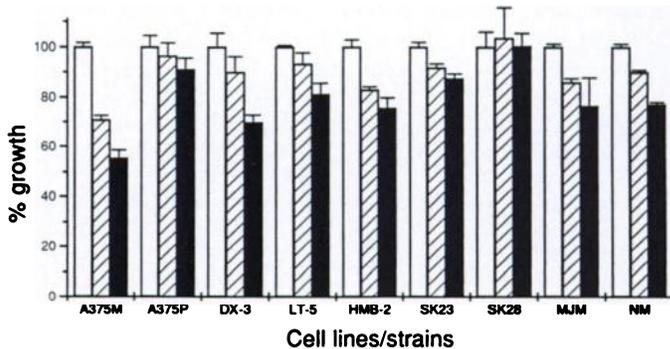


Fig. 5. Effect of two levels of IFN-α2b treatment on cell line proliferation, expressed as a percentage of control growth (i.e., in the absence of IFN-α2b). □, no IFN-α2b; ▨, 1,000 IU/ml IFN-α2b; ■, 10,000 IU/ml IFN-α2b. Bars, SD from the mean of triplicate wells.

ously, IFN- α had only a relatively small antiproliferative effect, if any, on either normal melanocytes or the majority of the melanoma lines, with the major effect occurring at the superphysiological concentration of 10,000 IU/ml. Line A375-M appeared the most sensitive to IFN- α , with an optimum reduction in cell growth to 55.4% of that occurring in the absence of IFN- α . The growth of the remaining cell lines was only reduced by approximately 20–30% (DX-3, LT-5, HMB-2, and normal melanocytes), 10% (A375-P and SK23), or not detectably (SK28). There is, therefore, no correlation between the ability of IFN- α pretreatment to induce activation of the IFN- α genes on SV infection and the degree of its antiproliferative effect on these cells *in vitro*.

DISCUSSION

The interferons were originally identified as proteins responsible for cellular resistance to viral infection. Evidence has accumulated, however, that indicates that IFNs may also play a part in protection against tumorigenic changes. A role for IFN- α and IFN- β in the development of malignant melanoma was hypothesized because of the therapeutic value of IFN- α in the treatment of this disease and the fact that IFN- α and IFN- β genes are found frequently to be deleted or rearranged in melanoma cells. A 40-kb region of chromosome 9 (within 9p21) has been elucidated recently as the minimum region of overlap of DNA deletions within melanoma cell lines. Although not encompassing the IFN genes, this region lies in close proximity to and just centromeric of this locus. It is possible, therefore, that this region includes *cis*-acting regulatory elements critical for transcription from the IFN- α and IFN- β loci. This type of wholesale gene family control is exemplified by the β -globin genes, where a dominant control region is located 54 kb upstream of the β -globin locus (26). Therefore, we examined the ability of both normal melanocytes and melanoma cells to transcribe and secrete IFN- α and IFN- β .

Normal human melanocytes were found to secrete both IFN- α and IFN- β in response to virus induction. Melanoma cell lines, however, were found to be defective in IFN expression. One of the melanoma cell lines examined, SK28, had lost the ability to secrete both IFN- α and IFN- β on virus induction. All of the six remaining melanoma lines, although retaining their ability to secrete IFN- β , had lost their ability to secrete IFN- α in response to SV induction (Table 1). This phenomenon was not due to deletion of all IFN- α genes, as shown by specific PCR amplification from genomic DNA (Fig. 1), nor was it due to the inability of the cells to efficiently translate IFN- α mRNA or secrete IFN- α proteins (Table 2).

On further examination, this loss of an IFN- α response was found to occur at the level of the promoter activation, since constructs containing the IFN- α promoter were completely inactive in these cells (Fig. 2). These results indicate that loss or mutation of a *cis*-acting dominant control region, analogous to β -globin gene control, is probably not responsible for the inability of the cells to produce IFN- α and suggests that there may be a dysfunction of a regulatory factor acting in *trans*. In contrast to the observations on IFN- α , only one melanoma cell line, SK28, had lost the ability to express IFN- β in response to SV induction (Table 1), and this loss did not appear to be due to lack of activity of the promoter (Fig. 3). This may be explained by a rearrangement or deletion of the IFN- β gene within this cell line, but this has not been examined in this study.

The significance of loss of IFN- α gene activity to the development of malignant melanoma is unclear. It is possible that this phenomenon is simply an artifact of cell culture, since only a minority of tumors form permanent lines upon explant, and adaptation of cells to culture has unknown genetic consequences. Unfortunately, it was not possible, in this study, to perform the IFN- α gene expression analysis on

freshly isolated melanoma cells due to a technical inability to isolate the melanoma cells from contaminating fibroblasts.

Whether the IFN- α gene regulatory factor is encoded within the critical region of deletion in 9p21 is unknown. Loss of viral induction of the IFN system was, however, reported for another 9p21-linked malignancy, acute lymphocytic leukemia. It was found with both malignant T-cell lines (45) and freshly isolated leukemic cells (46) that cells which had apparently normal IFN genes were incapable which had apparently normal IFN genes were incapable of producing either IFN- α alone or both IFN- α and IFN- β on SV induction. Promoter activity was not examined in these studies. In contrast to normal melanocytic cells, however, normal T cells secrete little if any IFN- α or IFN- β in response to virus induction (47). Therefore, for acute lymphocytic leukemia, deletion or inactivation of the IFN- α loci may be of less significance.

In contrast, virus-induced transcription from the IFN- α and IFN- β loci is observed from normal human melanocytes *in vitro* and, furthermore, has been detected specifically in the normal *in vivo* environment of the melanocytes (the basal layer of the epidermis). These facts indicate a possible physiological role for IFN- α s in the normal control of proliferation and differentiation of these cells. It is, therefore, possible that loss of IFN- α gene activity, as opposed to that of IFN- β or IFN- γ , has a distinct role to play in the pathogenesis of melanoma, such as a direct contribution towards escape from normal growth constraints or survival of neoplastic phenotypes.

The cytogenetic linkage of melanoma is heterogeneous, with a proportion of melanomas reported to be linked to abnormalities in chromosome 1 rather than 9. Whether a general loss of IFN- α gene activity is evident across the whole heterogeneous array of melanomas is unknown. As the control of IFN gene activation is complex, inactivation of any of the *trans*-acting factors positioned on a number of chromosomes may result in a malignant phenotype. This may offer an explanation for the genetic heterogeneity observed in malignant melanoma.

The results reported here have implications for the study of the control of IFN- α and IFN- β expression. The specific loss of IFN- α promoter activity implies differential control of gene transcription between IFN- α and IFN- β . It suggests that there may be a specific factor responsible for the control of IFN- α gene expression whose normal activity is disrupted in all of the melanoma lines investigated. Although there is considerable data available on the mechanisms of activation of IFN- β expression, very little is known about the activation of IFN- α expression. Transcription factors that bind to the regulatory element (VRE) of the IFN- β gene include IRF I, IRF II, and NF- κ B (reviewed in Ref. 39). IRF I appears to be critical for IFN- β gene expression, and IRF I expression is induced by IFN- β , viruses, and dsRNA. Nevertheless, IRF I overexpression is not enough to stimulate IFN- β expression. A second event is required, which takes place on induction with virus or dsRNA. Whether such a dual event is required for IFN- α expression is not known. The IFN- α regulatory element contains an IRF-binding site, but as yet, other transcription factors have not been identified. The IFN- α pretreatment restoration of virus-induced IFN- α gene transcription in LT-5 cells may signify that activation of IFN- α genes also requires at least two distinct factors/events. The melanoma lines used in this study may, therefore, be useful tools for the determination of the differential activity of the type I IFNs and the dissection of their respective pathways of induction.

The finding that IFN- α pretreatment reversed the inactivity of the IFN- α promoter in only one of the seven cell lines tested probably reflects the heterogeneous nature of the chromosomal lesions that develop in melanoma. This may also reflect the finding that only a minority of melanoma patients benefit from IFN- α therapy. It will be

interesting to correlate reversal of IFN- α promoter activity with therapeutic benefit in melanoma patients, and this may offer the opportunity to develop a much needed diagnostic indicator for IFN- α efficacy in patients.

Finally, much interest has been focused recently on a putative tumor suppressor gene, *CDKN-2* (p16), which has been located to the critical region of deletion on 9p21 (16, 17), and is, therefore, suspected of being the melanoma susceptibility gene. Recent investigation of familial and sporadic melanoma has revealed contradictory results regarding the frequency of *CDKN-2* mutation or loss (18, 19, 20). Therefore, whether *CDKN-2* is the melanoma susceptibility gene remains controversial. It is possible that the failure of the IFN- α system works in concert with the absence of the *CDKN-2* tumor suppressor to generate the malignant phenotype.

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