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Promoter Attenuation in Gene Therapy: Interferon-γ and Tumor Necrosis Factor-α Inhibit Transgene Expression

LIHUI QIN,[1,2] YAOZHONG DING,[2] DOMINIQUE R. PAHUD,[1] EUGENIA CHANG,[2,3] MICHAEL J. IMPERIALE,[2,4] and JONATHAN S. BROMBERG[1,2]

ABSTRACT

One of the major limitations to current gene therapy is the low-level and transient vector gene expression due to poorly defined mechanisms, possibly including promoter attenuation or extinction. Because the application of gene therapy vectors *in vivo* induces cytokine production through specific or nonspecific immune responses, we hypothesized that cytokine-mediated signals may alter vector gene expression. Our data indicate that the cytokines interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) inhibit transgene expression from certain widely used viral promoters/enhancers (cytomegalovirus, Rous sarcoma virus, simian virus 40, Moloney murine leukemia virus long terminal repeat) delivered by adenoviral, retroviral or plasmid vectors in vitro. A constitutive cellular promoter (β-actin) is less sensitive to these cytokine effects. Inhibition is at the mRNA level and cytokines do not cause vector DNA degradation, inhibit total cellular protein synthesis, or kill infected/transfected cells. Administration of neutralizing anti-IFN-γ monoclonal antibody results in enhanced transgene expression *in vivo*. Thus, standard gene therapy vectors in current use may be improved by altering cytokine-responsive regulatory elements. Determination of the mechanisms involved in cytokine-regulated vector gene expression may improve the understanding of the cellular disposition of vectors for gene transfer and gene therapy.

OVERVIEW SUMMARY

Transgene expression can be eliminated even in the presence of substantial amounts of vector DNA in the transduced cells, which suggests that mechanisms other tban the antigen-specific immune response may mediate non-cytodestructive events that determine the presence of transgene expression. Our data indicate that the cytokines interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) inhibit transgene expression from certain widely used viral promoters/enhancers (human cytomegalovirus immediate early, Rous sarcoma virus long terminal repeat, simian virus 40, Moloney murine leukemia virus long terminal repeat) delivered by adenoviral, retroviral, or plasmid vectors *in vivo*. Inhibition is at the mRNA level and cytokines do not cause vector DNA degradation, inhibit total cellular protein synthesis, or kill infected/transfected cells. Thus, cytokine-regulated promoter function rather than specific immune destruction could limit transgene expression. These results have significant implications for the construction of transfer vectors for human gene therapy because gene transfer vectors could be exposed to a cytokine-rich environment when they are administered *in vivo*.

INTRODUCTION

GENE THERAPY is a potentially important technique, permitting novel approaches to the treatment of disease (Crystal, 1995); however, there are limitations due to low-level and transient gene expression (Friedmann, 1996; Wilson, 1996). The use of strong, constitutive viral promoters does not usually achieve long-term transgene expression *in vivo*; and promoter extinction occurs (Scharfmann *et al.*, 1991; Challita and Kohn, 1994; Rettinger *et al.*, 1994), even in the presence of substantial amounts of vector DNA in the transduced cells (Dwarki *et al.*, 1995; Yao *et al.*, 1996). Using retroviral vectors, which integrate into the host genome and theoretically result in perma-

Departments of [1]Surgery, [2]Microbiology and Immunology, [3]Pediatrics, and [4]Comprehensive Cancer Center, University of Michigan, Ann Arbor, MI 48109.

nent transgene expression, indefinite gene expression is achieved *in vitro*, but only at low levels or not at all *in vivo* (Scharfmann *et al.*, 1991; Rettinger *et al.*, 1994). The immune response to adenoviral vectors by cytotoxic T lymphocytes (CTL) is a significant limitation to vector expression (Engelhardt *et al.*, 1994; Yang *et al.*, 1994; Dai *et al.*, 1995; DeMatteo et al., 1995); and while improved transgene expression can be achieved using less immunogenic E2 temperature-sensitive adenoviral vectors (Engelhardt *et al.*, 1994), immunosuppressants (Yang *et al.*, 1994; Dai *et al.*, 1995; DeMatteo *et al.*, 1995; Engelhardt *et al.*, 1994), or immunodeficient mice (DeMatteo *et al.*, 1995), expression is either not permanent or is still attenuated in comparison to initial levels. These findings suggest that other factors are responsible for determining transferred gene expression.

It is noteworthy that immune responses may alter transgene expression in other ways. For example, recent studies (Tsui *et al.*, 1995; Guidotti *et al.*, 1996) demonstrate that in transgenic mice which replicate the hepatitis B virus (HBV) genome, $CD8^+$ CTLs reduce cellular HBV mRNA by a non-cytotoxic mechanism. Analysis shows that interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), secreted by antigen-activated CTL, result in promoter-specific, post-transcriptional clearance of viral mRNA. These results suggest that antigen-specific or nonspecific cytokine responses initiate a series of intracellular signals that influence transcriptional and translational regulation and hence the level of transferred gene expression (Gribaudo *et al.*, 1995; Harms and Splitter, 1995). We hypothesize that cytokine-initiated signaling is a major determinant of transferred gene expression.

MATERIALS AND METHODS

Vectors

Viral and plasmid vectors encoding the LacZ reporter gene are shown in Table 1. The adenoviral vectors were all generated from adenovirus type 5 with an E1 region deletion. AdHCMVβ-gal (Csete *et al.*, 1994), with a human CMV immediate early 1 (HCMVie1) promoter, was provided by Dr. A. Shaked (University of Pennsylvania); AdRSVβ-gal (Stratford-Perricaudet *et al.*, 1992) and AdβAβ-gal (Grossman *et al.*, 1992), with a Rous sarcoma virus long terminal repeat (RSV-LTR) and β-actin promoter plus CMV enhancer, respectively, were obtained from the vector core of the University of Michigan Medical Center. There may be uncharacterized differences other than promoter regions among these vectors because they were constructed by *in vivo* homologous recombination in different laboratories. Plasmid vectors: pMP6Aβ-gal (Philip *et al.*, 1994) with HCMVie1 promoter was provided by Dr. M. Philip (Applied Immune Sciences Inc., Santa Clara, CA); pRSVβ-gal (Gorman *et al.*, 1982) with RSV-LTR was provided by Dr. G. Warr (Medical University of South Carolina); pCH110 (Hall *et al.*, 1983) with SV40 promoter was purchased from Pharmacia (Piscataway, NJ). Retroviral vector MGF-LacZ (Tahara *et al.*, 1992) with the Moloney murine leukemia virus long terminal repeat (MMLV-LTR) promoter was provided by Dr. H. Tahara (University of Pittsburgh).

Reporter gene expression and cytotoxicity assay

C2C12 myoblasts (Yaffe and Saxel, 1977) were infected with adenoviral vectors at a multiplicity of infection (moi) of 10–100 for 20 hr (which resulted in >75% of cells expressing the β-Gal activity determined by X-Gal staining), or transfected with 2 μg of plasmid per 5×10^5 cells by 30 μg of Lipofectamine Reagent (GIBCO-BRL, Grand Island, NY) in serum-free OPTI-MEM (GIBCO-BRL) for 5 hr (which resulted 5–10% of cells expressing β-Gal determined by X-Gal staining), or infected with a retroviral vector at a moi of 2 in the presence of 8 μg/ml of Polybrene for 20 hr (which resulted in >50% cells expressing β-Gal determined by X-Gal staining). Cells were then trypsinized, replated at 1×10^4–1×10^5 cells/cm^2, exposed to IFN-γ and/or TNF-α at various doses for 24–72 hr, and assayed for β-Gal activity, total cellular protein, and cell viability. The β-Gal activity was determined by *o*-nitrophenyl-β-D-galactopyranoside (ONPG) assay kit (Stratagene, La Jolla, CA), the total cellular protein was determined by Bradford assay (Bio-Rad, Hercules, CA), cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO) assay (Mosmann, 1983), cell proliferation was determined by [^{3}H]thymidine incorporation, and the cell cycle was assayed by propidium iodide (PI) (Sigma) assay (Noguchi, 1996). All assays samples were performed in triplicate and experiments repeated at least three times.

Northern and Southern blots

C2C12 myoblasts were infected with adenoviral vectors and treated with cytokines as described above. The cells were lysed, and RNA and DNA were isolated separately (Trizol reagent, GIBCO-BRL, Grand Island, NY). The RNA was run on a 1% formaldehyde gel and transferred to a nylon membrane (Bio-Rad, Hercules, CA). The DNA was denatured with 0.2 *N* NaOH and loaded on a nylon membrane. Hybridization was performed

TABLE 1. VECTORS

Vector	*Promoter (enhancer)*	*Reference*
Adenoviral vector		
AdHCMVβ-gal	HCMVie1	Csete *et al.* (1994)
AdRSVβ-gal	RSV-LTR	Stratford-Perricaudet *et al.* (1992)
AdβAβ-gal	β-actin (CMV)	Grossman *et al.* (1992)
Plasmid		
pMP6Aβ-gal	HCMVie1	Philip *et al.* (1994)
pRSVβ-gal	RSV-LTR	Gorman *et al.* (1982)
pCH110	SV40	Hall *et al.* (1983)
Retroviral vector		
MFG-LacZ	MMLV-LTR	Tahara *et al.* (1992)

with [^{32}P]dCTP random primer-labeled probes (Stratagene, La Jolla, CA) specific for β-Gal, β-actin, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes. The β-Gal probe was the 3.7-kb *Bam* HI–*Hin*d III fragment from pCH110 (Hall *et al.*, 1983); the β-actin probe was a 540-bp polymerase chain reaction (PCR) product between base pairs 25 and 565 generated from C57BL/6 genomic DNA (Alonso *et al.*, 1986); the GAPDH probe was a 294-bp reverse transcription (RT)-PCR product between base pairs 309 and 602 generated from C57BL/10J lymphocytes (Sabath *et al.*, 1990). For Southern blotting of cardiac allografts, whole transplanted hearts were explanted, homogenized in Trizol, DNA isolated, restricted with *Bam* HI, and Southern blotted with [^{32}P]dCTP random primer-labeled β-Gal probe for a 4-kb adenoviral fragment.

Western blot

Lysates from the treated cells were run on a 7.5% SDS-PAGE, transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA), blotted with a mouse monoclonal antibody (mAb) specific for adenovirus 72K DNA binding protein (Reich *et al.*, 1983), and horseradish peroxidase (HRP)-coupled sheep anti-mouse immunoglobulin G (IgG) (Amersham, Arlington Heights, IL). The signal was detected by using the ECL Western blotting system (Amersham) and exposure to X-ray film.

β-Gal expression in a murine heterotopic, nonvascularized cardiac transplantation model

Donor neonatal C57BL/6 mice (Harlan-Sprague-Dawley, Inc., Indianapolis, IN) were sacrificed, and whole hearts were removed and placed in a subcutaneous (s.c.) position of the ear pinnae of C57BL/6 recipients (Qin *et al.*, 1996) Five × 10^7 pfu of AdHCMVβ-gal or AdRSVβ-gal in 5 μl was directly injected into the graft at the time of transplantation. Anti-IFN-γ mAb (R4-6A2) (Spitalny and Havell, 1984) and isotype-matched control mAb Y13-259 (Furth *et al.*, 1982) hybridomas were purchased from the American Type Culture Collection (ATCC; Rockville, MD) and grown in culture; the culture supernatant was purified over protein G columns (Pharmacia-LKB, Piscataway, NJ). Purified mAbs were injected intravenously (i.v.) at 100 μg every other day for six doses. The ears with transplanted hearts were harvested at various time points after transplantation, quick frozen, embedded in O.C.T. (Miles Scientific, Naperville, IL), sectioned at 10 μm, collected onto gelatin-coated glass slides, fixed at room temperature in 0.25% glutaraldehyde in phosphate-buffered saline (PBS) for 30 min, rinsed three times in PBS for a total of 30 min, and incubated at 37°C overnight in 0.5 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) solution containing 5 m*M* potassium ferrocyanide, 5 m*M* potassium ferricyanide, 2 m*M* $MgCl_2$, 1 m*M* spermidine, 0.02% Nonidet P-40, and 0.01% sodium deoxycholate in PBS. After staining, the sections were fixed with 4% formaldehyde and counterstained with eosin (Qin *et al.*, 1996). Quantitative assay of intragraft reporter gene expression was performed using the luminescent β-Gal detection kit II (Clontech, Palo Alto, CA) according to the manufacturer's recommendations.

RESULTS

IFN-γ and TNF-α inhibit adenoviral vector reporter gene expression without killing transduced cells

To test whether cytokine-mediated signals could alter vector gene expression, resulting in gene attenuation or extinction, C2C12 myoblasts were infected with adenoviral vectors encoding the LacZ reporter under the control of HCMVie1 (AdHCMVβ-gal), RSV-LTR (AdRSVβ-gal), or β-actin (AdβAβ-gal) promoters, exposed to 30 U/ml of IFN-γ and/or TNF-α for 24 hr and assayed for β-Gal activity, total cellular protein, cell viability, cell proliferation, and cell cycling. The results in Table 2 demonstrate that viral promoters (HCMVie1 and RSV-LTR) were sensitive to the inhibitory effects of the

TABLE 2. CYTOKINES INHIBIT VIRAL BUT NOT CELLULAR PROMOTERS

Vectors	*Cytokines (30 U/ml)*	*β-Gal activity (U/mg of cellular protein)*	*Percent inhibition*	*Protein (mg/ml)*	*Percent inhibition*
AdHCMVβ-gal					
	—	21.7 ± 0.6	—	3.95 ± 0.60	—
	IFN-γ	16.5 ± 0.4	24.0	3.86 ± 0.60	3
	TNF-α	9.79 ± 0.29	54.9	4.13 ± 0.61	−5
	IFN-γ/IFN-α	6.07 ± 0.13	72.0	4.14 ± 0.61	−5
AdRSVβ-gal					
	—	2.89 ± 0.05	—	3.94 ± 0.60	—
	IFN-γ	1.57 ± 0.08	45.6	3.78 ± 0.59	4
	TNF-α	1.77 ± 0.09	38.6	4.06 ± 0.61	−3
	IFN-γ/TNF-α	0.66 ± 0.26	77.2	4.00 ± 0.61	−2
AdbAβ-gal					
	—	5.59 ± 0.41	—	3.93 ± 0.60	—
	IFN-γ	4.32 ± 0.20	22.7	3.96 ± 0.60	−1
	TNF-α	6.23 ± 0.74	11.4	4.00 ± 0.61	−2
	IFN-γ/TNF-α	5.20 ± 0.49	6.9	3.76 ± 0.59	4

C2C12 myoblasts were infected with indicated adenoviral vectors for 20 hr (which resulted in >75% of cells expressing the β-Gal activity determined by X-Gal staining), the cells were then exposed to 30 U/ml of IFN-γ and/or TNF-α for 24 hr, and assayed for β-Gal activity (expressed as units per mg of cellular protein) and total cellular protein (expressed as mg per milliliter of cell lysate from 2 × 10^6 infected C2C12). The experiment was performed six times with similar results.

cytokines, compared to the cellular promoter (β-actin). There was a slight inhibitory effect on the β-actin promoter by IFN-γ but not by TNF-α. It should be noted that this particular β-actin promoter construct also contained a CMVie enhancer (Grossmann *et al.*, 1992). Nonetheless, it was anticipated that a housekeeping gene promoter would be less sensitive to the regulatory effects of cytokines. Despite inhibition of β-Gal activity, total cellular protein was unaffected (Table 2), and there were no changes in cell viability, proliferation, and cycling (Fig. 1), showing that the cytokines did not have global inhibitory effects on basic cellular functions. Additional experiments showed that while IFN-γ and TNF-α had inhibitory effects on reporter expression, other cytokines, including pro-inflammatory cytokines [interleukin-1 (IL-1), IL-6, and TGF-β], TH1 cytokines (IL-2 and IL-12), TH2 cytokines (IL-4 and IL-10), and chemokines [macrophage inhibitory protein-1α (MIP-1α), MIP1β, MIP2, and KC] did not, even at high doses (not shown).

IFN-γ and TNF-α synergistically inhibit reporter gene expression from viral but not cellular promoters

More detailed dose–response analyses demonstrated that the HCMVie1 promoter was sensitive to the inhibitory effects of high concentrations of IFN-γ and very sensitive to even low doses of TNF-α. The RSV-LTR promoter was generally very sensitive to IFN-γ and less sensitive to TNF-α inhibitory effects. The β-actin promoter showed some sensitivity to only high concentrations of either IFN-γ or TNF-α (Fig. 2A,B). Whereas the β-actin and HCMVie1 promoters appear to have similar sensitivities to high concentrations of IFN-γ (Fig. 2A), differences are readily apparent at lower, physiologically relevant concentrations (Table 2; Fig. 2C). Importantly, the combination of IFN-γ plus TNF-α had marked synergistic inhibitory effects on the HCMVie1 and RSV-LTR promoters, but none did on the β-actin promoter (Fig. 2C). We conclude that IFN-γ and TNF-α initiate marked inhibitory and synergistic effects on selected viral promoters (HCMVie1, RSV-LTR) but not on a cellular promoter (β-actin) in adenoviral gene transfer vectors.

IFN-γ and TNF-α inhibit transgene expression at mRNA level without degrading viral DNA

To understand the level at which cytokines regulate reporter protein expression, Northern blotting was performed on cytokine-treated cells and showed that IFN-γ plus TNF-α synergistically decreased the level of β-Gal mRNA by about 60% from the HCMVie1 and RSV-LTR promoters, whereas they had much less effect on the β-actin promoter (Fig. 3A). It is interesting to note that IFN-γ and TNF-α had a similar magnitude of effect on reporter activity and mRNA expression (compare the percent in-

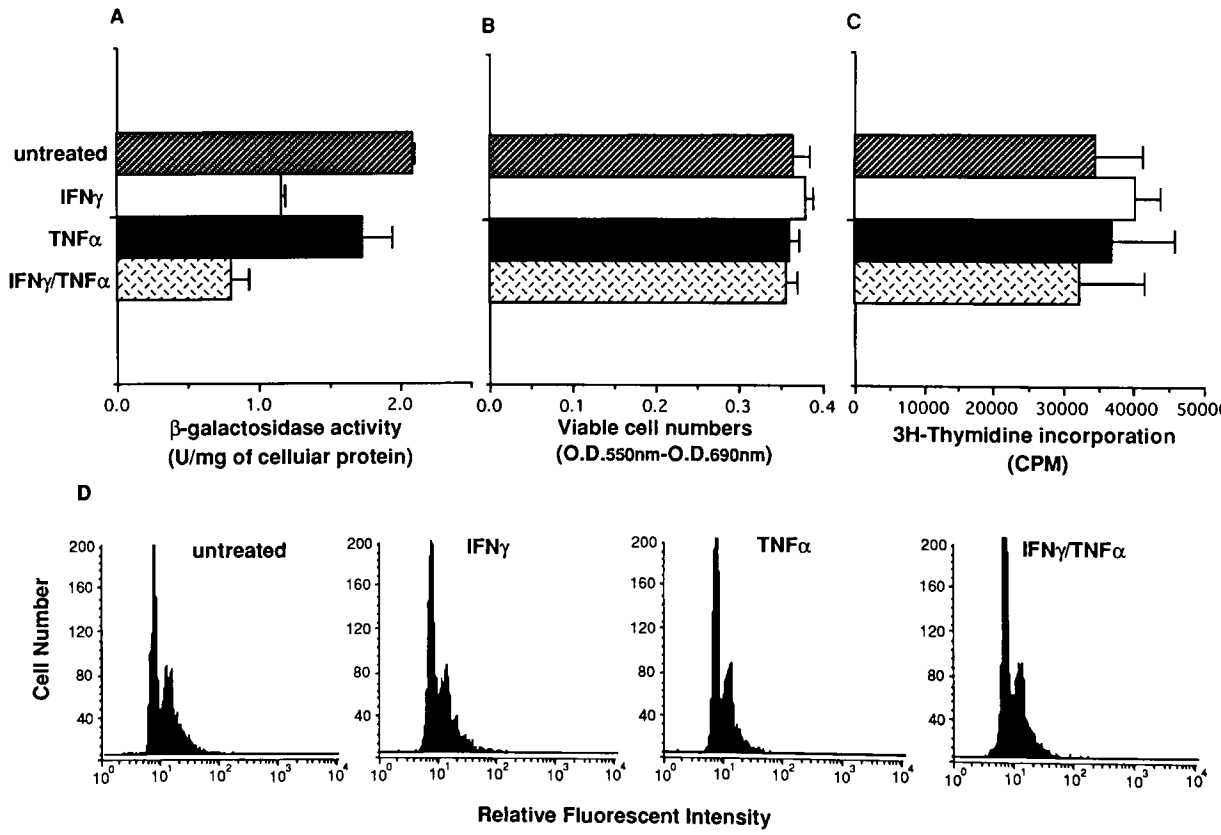

FIG. 1. IFN-γ and TNF-α inhibit reporter gene expression but do not alter cell viability, proliferation, and cycling. C2C12 myoblasts were infected with AdRSVβ-gal, treated with 30 U/ml of the indicated cytokines for 20 hr, and assayed for β-Galactosidase activity by ONPG assay (A), cell viability by MTT assay (B), cell proliferation by [^{3}H]thymidine incorporation (C), and cell cycle by PI assay (D).

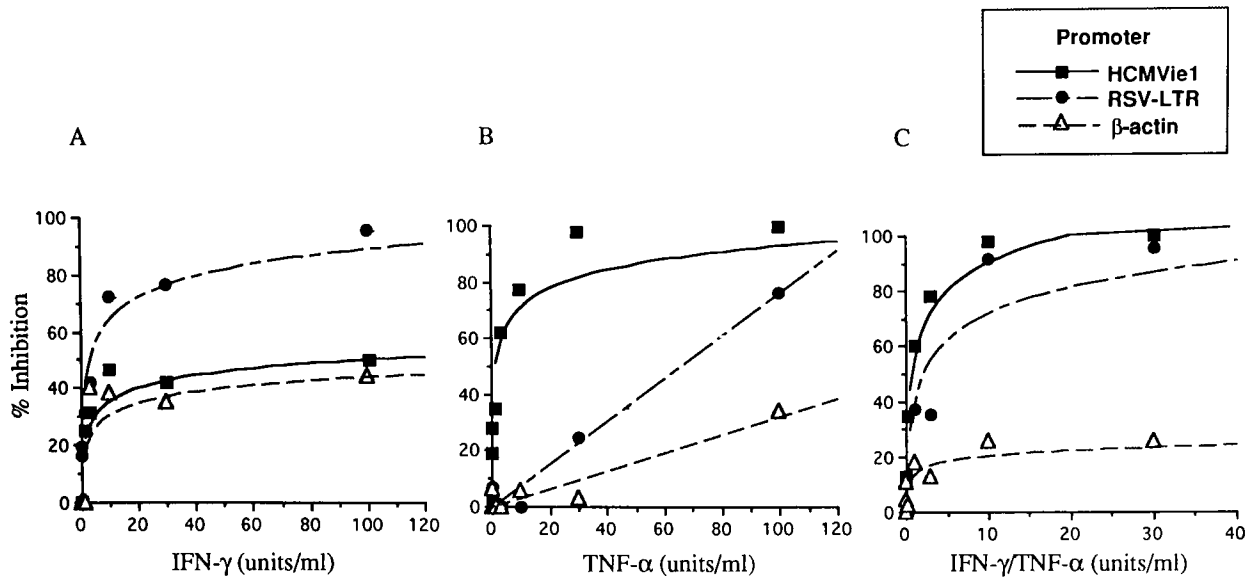

FIG. 2. IFN-γ (A) and TNF-α (B) synergistically inhibit vector gene expression. C2C12 myoblasts were infected with adenovectors encoding the LacZ reporter under the control of HCMVie1, RSV-LTR, or β-actin promoters. After overnight infection, cells were exposed to the indicated cytokines for 48 hr and then assayed for β-Gal activity by quantitative ONPG assay. Results are expressed as percent inhibition of control, untreated cultures. C. IFN-γ and TNF-α were administered in equal concentrations. The doses shown in the graphs were determined by MTT assay not to be cytotoxic. Higher doses of single or combined cytokines were cytotoxic (not shown). The experiment was performed five times with similar results.

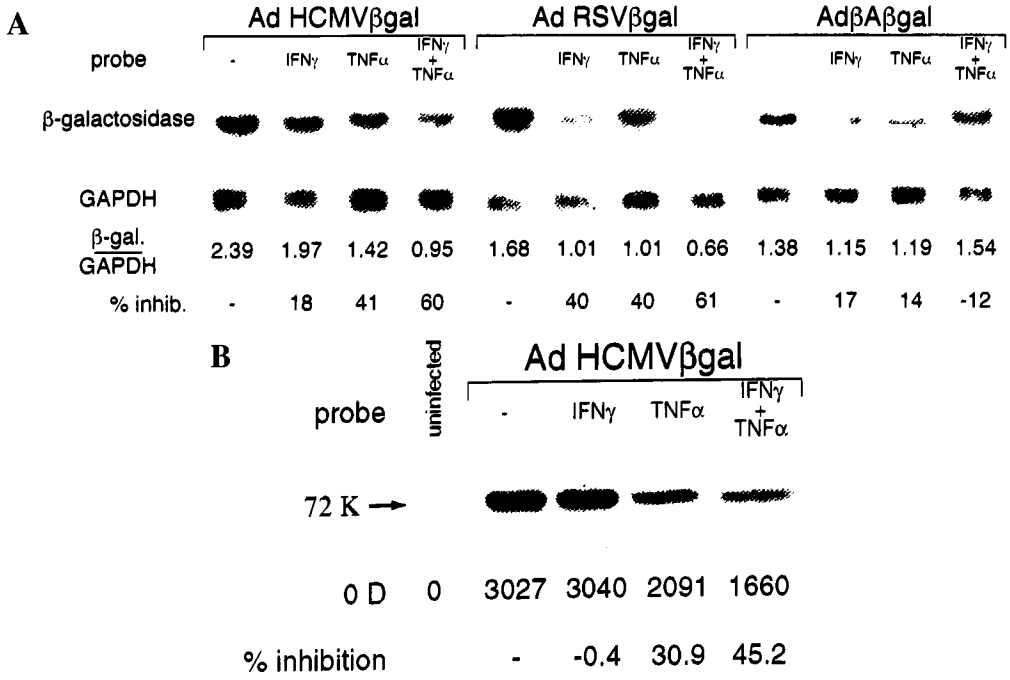

FIG. 3. A. IFN-γ and TNF-α synergistically inhibit mRNA expression from the HCMVie1 and RSV-LTR but not the β-actin promoter. C2C12 myoblasts were infected with the indicated adenoviral vectors, treated with 30 U/ml of cytokines for 20 hr, RNA was isolated, and Northern blotting was performed for β-Gal, β-actin, and GAPDH. Numbers represent the OD ratio of β-Gal: GAPDH and percent inhibition of expression. β-Actin expression showed a pattern identical to GAPDH expression (not shown). B. IFN-γ and TNF-α affect the expression of adenovirus 72K DNA binding protein. C2C12 myoblasts were infected with AdHCMVβ-gal, treated with cytokines at 30 U/ml for 20 hr, cell lysates run on 7.5% SDS-PAGE, and Western blotting performed for 72K DNA binding protein. Numbers represent the OD of the protein and percent inhibition of expression. The experiments were performed three times with similar results.

TABLE 3. CYTOKINES INHIBIT REPORTER GENE EXPRESSION FROM PLASMID VECTORS

Vector	*Promoter*	*Cytokines (30U/ml)*	*β-Gal activity (U/mg of cellular protein)*	*Percent inhibition*
pMP6Aβ-gal	HCMVie1	—	1.84 ± 0.01	—
		IFN-γ	1.47 ± 0.05	20.0
		TNF-α	1.30 ± 0.02	29.3
		IFN-γ/IFN-α	0.65 ± 0.04	64.7
pRSVβ-gal	RSV-LTR	—	0.83 ± 0.01	—
		IFN-γ	0.68 ± 0.01	18.1
		TNF-α	0.60 ± 0.01	27.7
		IFN-γ/TNF-α	0.51 ± 0.02	38.6
pCH110	SV40	—	0.44 ± 0.01	—
		IFN-γ	0.34 ± 0.01	23.7
		TNF-α	0.37 ± 0.01	15.9
		IFN-γ/TNF-α	0.29 ± 0.03	34.1

5×10^5 C2C12 cells were transfected with 2 μg of indicated plasmids encoding the LacZ reporter by 30 μg of Lipofectamine Reagent (GIBCO-BRL, Grand Island, NY) in serum-free OPTI-MEM (GIBCO-BRL, Grand Island, NY) for 5 hr, treated with 30 U/ml of IFN-γ and/or TNF-α for 24 hr, and assayed as described. The experiment was performed three times with similar results.

hibition in Table 2 versus Fig. 3A). This suggests that mRNA transcription is a major locus of control of gene expression by these cytokines. Southern blot analysis of vector DNA from these cells showed no difference among the groups (not shown). This suggests that IFN-γ and TNF-α are not activating cellular mechanisms that degrade the vector genome or otherwise rid the cells of the vector DNA. Lysates from these same cells were analyzed by Western blotting for the 72K DNA binding protein of adenovirus. The 72K DNA binding protein is normally expressed by these E1-deleted adenoviral vectors, controlled by the adenoviral E2 promoter 27 kb downstream from the reporter, and transcribed from the linear DNA genome in the opposite direction from the reporter gene (Reich *et al.*, 1983). The Western blotting results (Fig. 3B) demonstrated that IFN-γ had no effect on 72K DNA binding protein expression, whereas TNF-α decreased expression by about 30% and the combined IFN-γ plus TNF-α treatment decreased expression by about 45%. This shows that cytokines may affect not only gene expression controlled by the HCMVie1 promoter but also expression of other genes on the same adenoviral genome. Because the cytokine effects on reporter activity and on 72K DNA binding protein expression are not the same (compare the percent inhibition in Fig. 3B versus Table 2 and the effect of IFN-γ versus TNF-α), this suggests that cytokines initiate signals that regulate different viral promoters in a promoter-specific fashion.

IFN-γ and TNF-α inhibit transgene expression delivered by plasmid and retroviral vectors

To determine if these cytokine inhibitory effects were associated only with adenoviral vectors or could be observed with other vectors, C2C12 cells were transfected with plasmid DNA encoding the LacZ reporter, or infected with a retroviral vector encoding LacZ, treated with IFN-γ and/or TNF-α, and assayed as described above. The results in Table 3 demonstrate that IFN-γ and TNF-α were able to inhibit plasmid reporter expression from HCMVie1, RSV-LTR, and SV40 promoters and did so in an additive or synergistic fashion. These results are qualitatively similar to those with adenoviral vectors, and controls likewise showed no effect on cell viability or proliferation (not shown). IFN-γ and TNF-α also inhibited reporter gene expression from the retroviral vector MFG-LacZ with the MMLV-LTR promoter (Table 4). Transgene expression could be restored two days after these cytokines were removed from culture (Table 4), which again indicates that inhibition of gene expression is at the transcriptional and/or translational level and that cytokines do not cause vector nucleic acid degradation or kill transduced cells. These results further support the hypothesis that cytokine-initiated events are acting at the level of transcriptional control and do not involve other vector or virus-specific loci or mechanisms.

Anti-IFN-γ mAb prolongs adenoviral vector reporter gene expression in vivo

To determine if cytokine manipulation was also relevant for the expression of transferred genes *in vivo*, syngeneic cardiac transplants were performed using C57BL/6 donor hearts and recipient mice. The operative trauma and ischemic injury to the graft result in tissue damage and immunologically nonspecific generation of cytokines such as IFN-γ. The grafts were each injected with the viral vector AdHCMVβ-gal. Recipients were also untreated or treated with anti-IFN-γ mAb or an isotype-matched control mAb, grafts harvested at various time points after transplantation, and frozen sections stained with X-Gal for β-Gal activity. The treatment with adenoviral vectors or mAbs did not affect the survival of the syngeneic grafts as determined by electrocardiogram (EKG) verification (not shown). The results in Fig. 4, however, demonstrate that anti-IFNγ mAb treatment resulted in more prolonged and higher level β-Gal expression, as determined by X-Gal staining, to at least 30 days after transplantation, suggesting enhanced reporter gene expression as a result of neutralizing IFN-γ with specific mAb.

Similar results were observed using a quantitative β-Gal assay. Reporter gene expression from AdRSVβ-gal could be prolonged by anti-IFN-γ mAb (Fig. 5) to a level 10 times higher

TABLE 4. CYTOKINES INHIBIT REPORTER GENE EXPRESSION FROM A RETROVIRAL VECTOR

Group	Cytokines (30U/ml)	β-Gal activity (U/mg of cellular protein)	Percent inhibition	Protein (mg/ml)	Percent inhibition
I. Cells treated with cytokines for 20 hr					
	—	1.50 ± 0.02	—	2.94 ± 0.10	—
	IFN-γ	0.33 ± 0.03	78.0	2.94 ± 0.40	0
	TNF-α	1.00 ± 0.08	33.3	2.94 ± 0.44	0
	IFN-γ/IFN-α	0.06 ± 0.03	96.0	2.86 ± 0.15	2.7
II. Cells treated with cytokines for 3 days					
	—	1.57 ± 0.01	—	8.88 ± 1.07	—
	IFN-γ	0.45 ± 0.02	71.3	8.91 ± 1.07	−0.3
	TNF-α	0.60 ± 0.01	59.9	8.50 ± 1.05	4.3
	IFN-γ/TNF-α	0.08 ± 0.01	95.1	7.98 ± 1.03	10.1
III. Cells treated with cytokines for 20 hr, washed and incubated for additional 2 days					
	—	1.09 ± 0.01	—	9.30 ± 1.09	—
	IFN-γ	0.90 ± 0.01	17.1	9.25 ± 1.08	0.5
	TNF-α	1.00 ± 0.02	7.9	9.31 ± 1.09	−0.1
	IFN-γ/TNF-α	1.10 ± 0.04	−0.9	8.68 ± 1.06	6.7

C2C12 cells were infected with a retroviral vector MFG-LacZ at moi of 2 in the presence of 8 mg/ml of Polybrene (which resulted >50% cells expressing β-Gal determined by X-Gal staining). Transduced cells were treated with 30 U/ml of IFN-γ and/or TNF-α for (I) 20 hr, (II) 3 days, and (III) 20 hr followed by washing and incubation for an additional 2 days, and assayed as described. The experiment was performed three times with similar results.

than control. These results are commensurate with those obtained with the C2C12 myoblast line *in vitro*.

It is possible that these results could be explained not only by differences in promoter function as a result of neutralizing IFN-γ with specific mAb, but also by inhibition of anti-adenoviral vector immunity and cytotoxicity by the mAb. To address this issue, grafts were harvested 10 days after transplantation, a time at which there was a large difference in X-Gal staining between control and treated groups, as shown in Fig. 4. DNA was isolated, digested with *Bam* HI, and Southern blotted with the β-Gal probe. The results demonstrate no difference between the groups in the amount of vector DNA in the cardiac tissues (Fig. 6). This suggests that the difference in reporter expression is due to the effects of promoter activity on gene transcription and not due to the specific immune response killing infected cells, which would have caused a decrease in vector DNA and affected syngeneic graft survival, neither of which effects were observed.

DISCUSSION

The data here indicate that the cytokines IFN-γ and TNF-α inhibit transgene expression from certain widely used viral promoters (CMV, RSV, SV40, MMLV-LTR) delivered by adenoviral, retroviral, or plasmid vectors *in vitro*. Inhibition is at the mRNA level and cytokines do not cause vector DNA degradation, inhibit total cellular protein synthesis, or kill infected/transfected cells. It is possible that these cytokine effects have not generally been noted previously because most

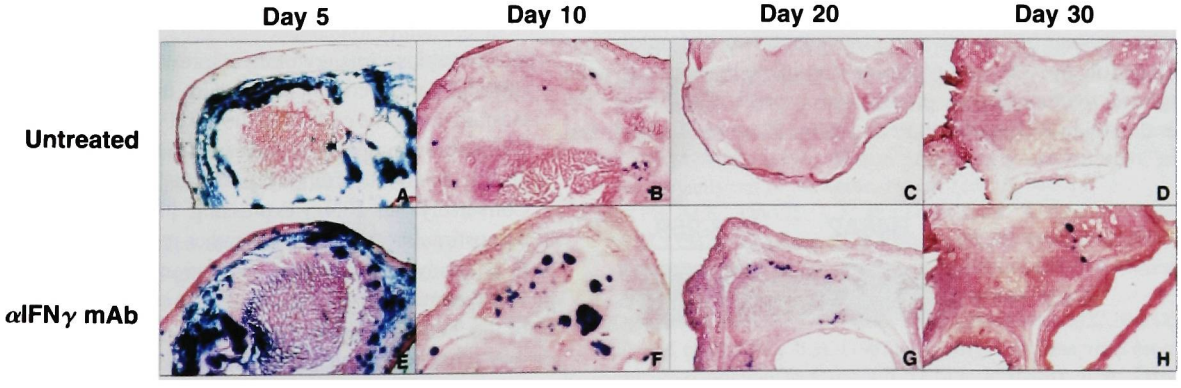

FIG. 4. Anti-IFN-γ mAb prolongs adenoviral vector reporter gene expression. Syngeneic cardiac transplants were performed and grafts were injected with 5×10^7 pfu of AdHCMVβ-gal. One group of recipients received no mAb (A–D), while a second group was treated with 100 μg of anti-IFN-γ mAb R4-6A2 i.v. on days 0, 2, 4, 6, 8, and 10 with respect to transplantation (E–H). Grafts were harvested on days 5 (A and E), 10 (B and F), 20 (C and G), and 30 (D and H) after transplantation and stained with X-Gal for β-Gal activity. Original magnification, 100×. There were at least three grafts for each group at each time point. Groups treated with control Y13-259 mAb were no different from the untreated controls (not shown).

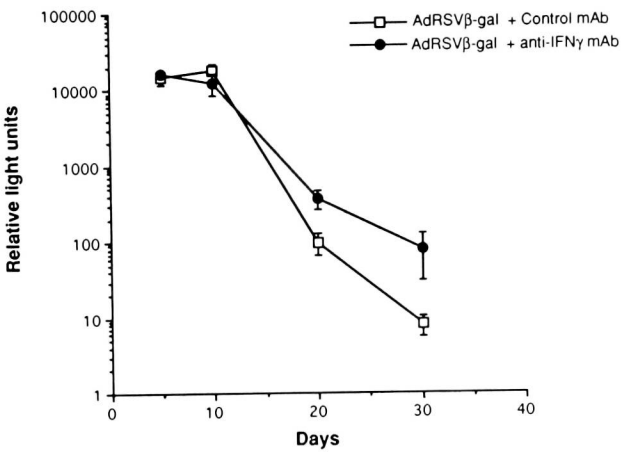

FIG. 5. Anti-IFN-γ mAb prolongs adenoviral vector reporter gene expression. Syngeneic cardiac transplants were performed and grafts were injected with 5×10^7 pfu of AdRSVβ-gal. Recipients were treated with 100 μg of anti-IFN-γ mAb or control mAb on days 0, 2, 4, 6, 8, and 10 with respect to transplantation. Grafts were harvested 5, 10, 20, and 30 days after transplantation and assayed for β-Gal activity by luminescent β-Gal detection. Note that results are given on a logarithmic scale.

work has been performed *in vitro* under conditions of low and unchanging cytokine concentrations. These results have significant implications for the construction of transfer vectors because there are at least three situations in which gene transfer vectors could be exposed to a cytokine-rich environment when they are administered *in vivo*: (i) Gene therapy has been considered for the treatment of diseases (*e.g.*, cancer, AIDS, transplantation, atherosclerosis, autoimmunity, ischemia-reperfusion) (Crystal, 1995), which have in common the ubiquitous production of cytokines through either antigen-specific stimulation of lymphocytes, or nonspecific stimulation of lymphocytes, other leukocytes, vascular endothelial cells, or organ parenchymal cells by infection, ischemia, necrosis, or trauma. (ii) Gene therapy protocols frequently involve gene transfer of cytokines to either elicit immune responses (*e.g.*, cancer) or inhibit immune responses (*e.g.*, transplantation and autoimmunity). (iii) Specific and nonspecific immune responses to gene transfer vectors or gene products are capable of inducing several cytokines (Engelhardt *et al.*, 1994; Yang *et al.*, 1994; Dai *et al.*, 1995; DeMatteo *et al.*, 1995; Krieg *et al.*, 1995; Sato *et al.*, 1996). For example, recombinant adenoviruses stimulate host immune responses which involve IFN-γ and IL-2 (Engelhardt *et al.*, 1994; Yang *et al.*, 1994; Dai *et al.*, 1995; DeMatteo *et al.*, 1995). In addition, the transferred nucleic acids themselves are capable of inducing several cytokines including IFN-α, IFN-β, IL-12, and IFN-γ (Krieg *et al.*, 1995; Sato *et al.*, 1996). Despite the fact that cytokines are likely to be frequently present during gene therapy applications and influence cellular responses, there are few studies in the current gene therapy literature that examine the effects of cytokine signals on the transcriptional and translational disposition of transferred genes (Harms and Splitter, 1995). Instead, the cellular targets of gene transfer vectors and the other physiologic signals impinging on these cells have often been considered passive participants in the process of gene transfer and expression.

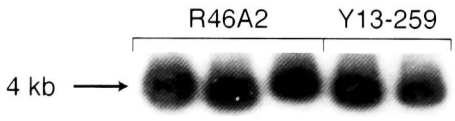

FIG. 6. Anti-IFN-γ mAb does not affect the persistence of adenoviral genomic DNA in cardiac transplants. Recipients of syngeneic cardiac grafts infected with 5×10^7 pfu of AdHCMVβ-gal received anti-IFN-γ (R4-6A2) or control mAb (Y13-259) as detailed in the Materials and Methods section. The grafts were explanted after 10 days, and Southern blotting for β-Gal was performed on adenoviral genomic DNA isolated from the cardiac tissues.

It is notable that the CMVie, RSV, SV40, and MMLV-LTR promoters and enhancers, which are cytokine sensitive as shown here, are widely used in gene transfer vectors. Thus, vectors in current use may not be entirely appropriately constructed to provide for sustained gene expression because the cytokine environment or the gene transfer product itself may inhibit its own promoter. For example, IFN-γ and TNF-α have been transduced into tumor cell lines or tumor-infiltrating lymphocytes by retroviral vectors with the MMLV-LTR promoter (Karp *et al.*, 1993; Marincola *et al.*, 1994). Much of the work has focused on characterizing short-term gene expression of cells transduced *in vitro* rather than evaluating chronic gene expression in conjunction with potential antitumor activity *in vivo*. In fact, genetically modified, TNF-α-secreting, nonimmunogenic murine fibrosarcomas failed to elicit a host immune response, which is in contrast to the effects with an IL-2-secreting tumor (Karp *et al.*, 1993). In other applications of gene therapy, retroviral vectors are used to transduce bone marrow stem cells, which are then expanded in the presence of growth factors, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), stem cell factor (SCF), IL-3, IL-6, and IL-7 (Lu *et al.*, 1993), yet the effects of these growth factors on viral promoters or other promoters have not been investigated.

In an attempt to enhance the efficacy of a plasmid DNA vaccine to rabies virus, cytokine-expressing plasmids (IFN-γ and GM-CSF) were co-inoculated with the viral antigen encoding plasmid under the control of the SV40 early promoter. The GM-CSF-expressing plasmid increased antiviral immunity by stimulating both the antibody and the T helper cell responses. Conversely, an IFN-γ-expressing plasmid had no such immunostimulatory effects, but rather resulted in a slight decrease in antibody and cytokine production (Xiang and Ertl, 1995). This unanticipated effect of IFN-γ could be due to inhibition of the SV40 early promoter by IFN-γ, so that less viral antigen was produced when the plasmid was co-injected with IFN-γ-expressing plasmid. T lymphocytes are particularly recalcitrant to attempts at stable gene transfer and expression (Rosenberg, 1991; Chu *et al.*, 1992). Because these cells are immunologically active, and in particular produce IFN-γ and TNF-α, the results presented here may provide a partial explanation for this resistance. It should also be noted that these and other cytokines can activate these viral promoters under other circumstances

(Tzen and Scott, 1993; Stein *et al.*, 1993; Fietze *et al.*, 1994; John *et al.*, 1994 Laegreid *et al.*, 1994); and that these promoters can occasionally direct long-term gene expression *in vivo*, even in immunocompetent recipients (Barr *et al.*, 1995; Poller *et al.*, 1996).

It is well known that IFNs, produced by virus-infected cells as part of the innate immune system, can signal to neighboring cells to resist viral replication and prevent virus spread (Samuel, 1991; Farrar and Schreiber, 1993). TNF-α is also a potent antiviral cytokine that acts alone or in a synergistic manner with IFN-γ to protect cells from viral infection (Wong *et al.*, 1986, 1992; Wietzerbin *et al.*, 1990). A number of studies suggest that these cytokines are basic cellular defenses against viral nucleic acids and that cells can distinguish viral from cellular sequences. For example, it has been reported that IFN-γ and IFN-α can inhibit the transcription of murine cytomegalovirus (MCMV) immediate-early genes (Gribaudo *et al.*, 1995). The inhibitory effect on MCMVie gene enhancer function is related at least partially to cytokine-induced inhibition of NF-κB activity (Gribaudo *et al.*, 1995). It is likely that NF-κB activity is directly inhibited by the p202 protein, which is induced by IFN-γ and which directly binds to and inhibits the function of the p50 and p65 subunits of NF-κB (Wang *et al.*, 1996). p202 can also bind to and inhibit the c-fos and c-jun subunits of the AP-1 transcription factor (Wang *et al.*, 1996), which may have negative regulatory effects because AP-1 binding sites have been mapped inside the MCMV promoter/enhancer region (Gribaudo *et al.*, 1995). Because p202 inhibits cell cycling and proliferation, it is probably not mediating the effects observed in the present report because the cytokines did not affect C2C12 proliferation. A more recent report shows that IFN-γ-induced STAT1 competes for the CBP and p300 co-activator proteins, and thereby inhibits AP-1 and ets-dependent transcriptional activation (Horvai *et al.*, 1997). There is also recent evidence that Kupfer cells, known producers of IFN-γ and TNF-α, play a role in immunologically nonspecific elimination of adenoviral vectors in the liver following *in vivo* administration (Worgall *et al.*, 1997). Inhibition of early viral promoters, as shown here, provides an additional mechanism for the antiviral effects of IFNs and TNF-α. Determination of the mechanisms involved in cytokine-regulated gene expression and a comprehensive understanding of promoter cytokine sensitivities may significantly alter the design of vectors for gene transfer and gene therapy.

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Address reprint requests to:
Dr. Jonathan S. Bromberg
Department of Surgery
University of Michigan
2926 Taubman Center
Ann Arbor, MI 48109-0331

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