JOURNAL OF VIROLOGY, Dec. 1997, p. 9163–9169 Vol. 71, No. 12
0022-538X/97/$04.00+0

Repression of Retrovirus-Mediated Transgene Expression by Interferons: Implications for Gene Therapy

SOOSAN GHAZIZADEH,[1]* JOSEPH M. CARROLL,[2]† AND LORNE B. TAICHMAN[1]

Department of Oral Biology and Pathology, State University of New York at Stony Brook, Stony Brook, New York 11794,[1] and Keratinocyte Laboratory, Imperial Cancer Research Found, London, United Kingdom[2]

Received 30 May 1997/Accepted 19 August 1997

Retrovirus-mediated gene transfer is commonly used in gene therapy protocols and has the potential to provide long-term expression of the transgene. Although expression of a retrovirus-delivered transgene is satisfactory in cultured cells, it has been difficult to achieve consistent and high-level expression in vivo. In this investigation, we explored the possibility of modulating transgene expression by host-derived cytokines. Normal human keratinocytes and dermal fibroblasts were transduced with recombinant retroviruses expressing a reporter gene (*lacZ*). Treatment of transduced cells with a proinflammatory cytokine, gamma interferon (IFN-γ), significantly reduced *lacZ* expression to less than 25% of that of nontreated cells. The inhibition was concentration dependent (peak at 5 ng/ml) and time dependent (maximal at 16 h for transcript and 24 h for protein); expression remained repressed in the continued presence of IFN-γ but returned to normal levels 24 h after IFN-γ withdrawal. The decrease in β-galactosidase activity appeared to result from decrease in steady-state *lacZ* mRNA levels. Inhibitors of transcription and translation blocked IFN-γ-induced repression, suggesting involvement of newly synthesized protein intermediates. Similar results were obtained by treatment of transduced cells with IFN-α but not with other proinflammatory cytokines, including tumor necrosis factor alpha, interleukin-2 (IL-1), IL-4, and granulocyte colony-stimulating factor. Although the level of *lacZ* mRNA was reduced by >70% following IFN treatment, the rate of *lacZ* transcription was not significantly different from that for nontreated cells. These results suggest that IFN-mediated regulation of transgene expression is at a posttranscriptional level. Interestingly, IFN-γ also suppressed transgene expression driven by a cellular promoter (involucrin) inserted in an internal position in the retroviral vector. The presence of the overlapping 3′ untranslated regions in transcripts initiated from the internal promoter and the long terminal repeat is suggestive of a posttranscriptional regulation, likely at the level of RNA stabilization. These results provide direct evidence for modulatory effects of IFNs on retrovirus-mediated transgene expression and suggest that gene therapy results may be altered by host inflammatory responses.

Recombinant retroviral vectors based on Moloney murine leukemia virus (MMLV) remain the predominant means of gene transfer in gene therapy protocols (37). This is due to their well-characterized biology and high efficiency of transgene integration into the host chromosome. In addition, the viral promoter in the long terminal repeat (LTR) region is constitutively active in many cultured cell types (33). However, when normal cells are transduced and transplanted to animals, transgene expression is usually reduced, often to undetectable levels. Loss of gene expression from the transduced gene has been observed in several systems, including hematopoietic cells (3, 47), hepatocytes (25, 49), fibroblasts (39, 46), and keratinocytes (4, 14, 16, 36, 48). Poor expression in vivo has often been attributed to a specific decrease in transcription of the transferred gene once the transduced cells are grafted to the animal (3, 26). Selective methylation of proviral sequences has been proposed as one of the possible mechanisms of inactivation (3, 21, 43); however, it is unclear whether methylation is a primary or secondary event. The specifics of vector design and transduction protocols as well as the immune responses of the host may also contribute to the observed problems in transgene expression (40, 44).

One aspect of the in vivo environment that has not been explored for its effects on transgene expression is proinflammatory cytokines. As transplantation of ex vivo-modified cells to the host usually involves some surgical or traumatic intervention, it is likely that inflammatory reactions develop at the graft site. Several proinflammatory cytokines such as gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) have been shown to regulate both viral and cellular gene expression (6, 32) and, in the case of a transduced gene, could act directly or indirectly to alter transgene expression. The present study analyzes the effect of several proinflammatory cytokines on LTR-directed expression of a transduced gene in normal epidermal keratinocytes and dermal fibroblasts. We demonstrate that both IFN-γ and IFN-α down modulate expression of the retrovirus-delivered transgene. Interestingly, this down modulation also occurs with an internal cellular promoter whose expression is not known to be affected by the cytokines.

MATERIALS AND METHODS

Cells. Primary keratinocytes were obtained from human newborn foreskin and grown in submerged cultures in the presence of irradiated 3T3 cells (42), using keratinocyte medium described by Wu et al. (52) supplemented with 5% fetal bovine serum. Human dermal fibroblasts were the outgrowth of skin biopsies grown in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Retroviral vectors and virus-producing cells. The MFG retroviral vector containing the *Escherichia coli lacZ* reporter gene (MFG-LacZ) and the Ψ-CRIP amphotropic producer line were kindly provided by Richard Mulligan (24). Retroviral vector construct pBabe-gal was generated by subcloning a fragment containing the entire coding sequences of *E. coli lacZ* gene in the *Eco*RI/*Sal*I site

* Corresponding author. Mailing address: Department of Oral Biology and Pathology, SUNY at Stony Brook, Stony Brook, NY 11794-8702. Phone: (516) 632-8926. Fax: (516) 632-9707. E-mail: sghazizdeh@epo.som.sunysb.edu.

† Present address: Genetics Institute, Andover, MA 01810.

of pBabe (35). A DNA fragment containing nucleotides (nt) −2473 to +40 of the human involucrin promoter was cloned upstream of the *lacZ* gene to create pBabe-inv-gal (2). Retroviral vector constructs were transfected into the ecotropic packaging cell line Ψ-CRE (9) by the calcium phosphate coprecipitation method. Forty-eight hours later, the supernatant was used to transinfect the GP+envAm12 amphotropic packaging line (28). Colonies of infected cells were isolated by selection in the presence of medium containing puromycin (2.5 μg/ml; Sigma). Clones containing the full-length virus, as determined by Southern analysis, were expanded and used. Viral titer was determined on NIH 3T3 cells, and the supernatant of transduced cells was tested for the presence of helper virus by using a marker rescue assay (46).

Primary keratinocytes and fibroblasts were seeded at 2×10^5 cells per 10-cm dish and transduced 24 h later with 2 ml of virus-containing medium that had been filtered through 0.45-μm-pore-size filter (Gelman) and supplemented with Polybrene (8 μg/ml; Sigma) for 2 to 3 h at 37°C. Fresh medium was added; 2 days after infection, the cells were passaged, and one representative plate was fixed and stained with 4-chromo-5-bromo-3-indolyl-β-D-galactoside (X-Gal; Sigma) for β-galactosidase (β-Gal) activity. When pBabe viruses were used, cells were passed into the puromycin-containing medium (1 μg/ml), and selected pools of resistant cells were expanded and used.

IFN treatment and β-Gal assay. Cells were plated in 24-well plates at 2.5×10^4 cells per well and 24 h later treated with the cytokine recombinant human TNF-α, interleukin-1 (IL-1), IL-4, granulocyte colony-stimulating factor, IFN-γ (2.7×10^7 IU/mg; Genzyme), or IFN-α (10^6 IU/ml) for 40 h at various doses. Cells were washed twice in cold phosphate-buffered saline and lysed in 30 μl of 0.25 M Tris (pH 7.8) by three successive freeze-thaw cycles. Protein concentrations of the lysates in representative wells were measured by using the bicinchoninic acid reagent (Pierce). β-Gal enzyme activity in lysates was determined by hydrolysis of 1 mg of o-nitrophenyl β-D-galactopyranoside (ONPG) per ml in 300 μl of sodium phosphate buffer containing 1 mM $MgCl_2$ and 45 mM β-mercaptoethanol at 37°C for 20 min. The reaction was stopped by addition of 500 μl of 1 M Na_2CO_3, and the absorbance was measured at 420 nm (at the linear range 0.2 to 0.8). Data are expressed as the relative absorbance of treated samples to untreated samples. Experiments were done in quadruplicate.

RNA analysis. Total RNA was isolated from cells by modified guanidinium isothiocyanate method (5). For keratinocytes, 3T3 feeders were removed with 5 mM EDTA prior to RNA extraction. Steady-state mRNA levels were quantified by hybridization of total RNA to specific ^{32}P-labeled antisense RNA probes in an RNase T_2 protection assay (31). Protected fragments were analyzed on a 5 to 6% polyacrylamide–8 M urea gel, and excised bands were quantified in a liquid scintillation counter. Antisense RNA probes of 635 nt for *lacZ* (generated by cloning the *Mul*I-*Sac*I [+2257 to +2892] fragment of *lacZ* coding sequences in pGEM3) and 270 nt for β-actin were used. The latter served as internal control for both repression and gel loading. The RNA probe used to differentiate LTR-initiated from involucrin promoter-initiated transcripts contained 323 nt of the involucrin promoter, including the first exon and 185 nt of contiguous *lacZ* coding sequences, and was generated by reverse transcription-PCR using RNA from pBabe-inv-gal-transduced keratinocytes. This fragment was subcloned in an RNA expression vector (pCRII; Invitrogen) and linearized with *Afl*II (cuts at −136 of the involucrin promoter), and T7 RNA polymerase was used to generate a 368-nt antisense RNA probe.

Determination of transcription rate. Nuclei were isolated by a technique described by Marzluff and Huang (29) from nontreated or cells treated with 5 ng of IFN-γ per ml for 12 h and frozen at −70°C. An additional aliquot of IFN-γ was added 2 h prior to isolation of nuclei to demonstrate transcriptional induction of the IFN regulatory factor gene (IRF-1), whose transcription is transiently induced following IFN-γ treatment (34). Thawed nuclei were incubated in a reaction buffer containing 25 mM Tris (pH 7.5), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 200 mM KCl, 25% glycerol, 500 μM each ATP, CTP, and GTP, and 300 μCi of [α-^{32}P]UTP (3,000 mCi/mmol) for 30 min at 30°C. Elongated RNA samples were purified and prepared for hybridization by precipitation with trichloroacetic acid. Five micrograms of plasmid DNA containing *lacZ* was used as probe for *lacZ* transcripts, and the backbone vector was used as a negative control. To avoid binding of *lacZ* transcript to *lacZ* sequences included in the Bluescript plasmid vector containing IRF-1 and actin cDNAs, the full-length IRF-1 and β-actin genes were isolated, and 1 μg of each was fixed to a nylon membrane by using a dot blot apparatus. Filters were hybridized with the labeled RNA samples at 10^7 cpm/blot. Autoradiography was performed for 3 to 5 days at −70°C, using X-ray film with an intensifying screen.

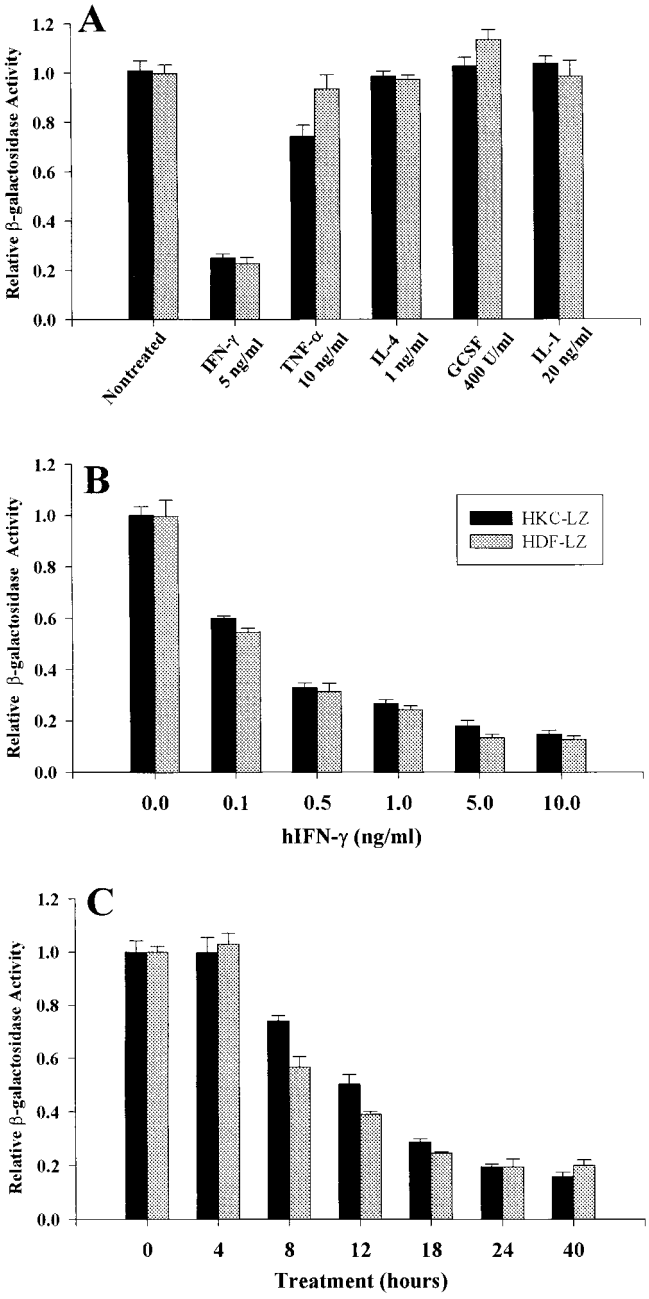

FIG. 1. Suppression of β-Gal activity in retrovirus-transduced cells treated with IFN-γ. (A) MFG-lacZ-transduced human keratinocyte (HKC-LZ) or dermal fibroblast (HDF-LZ) cultures were treated for 40 h with cytokines as indicated. GCSF, granulocyte colony-stimulating factor. (B) Cells were treated with graded concentrations of IFN-γ (ranging from 0.1 to 10 ng/ml) for 40 h. (C) Cells were treated with IFN-γ (5 ng/ml) for the times indicated at the bottom. β-Gal activity of the cell lysate was measured by using ONPG as the substrate. Experiments were performed in quadruplicate, and results are shown as mean ± standard error from three independent experiments.

RESULTS

Suppression of LTR-directed *lacZ* expression in retrovirus-transduced fibroblasts and keratinocytes treated with IFN-γ. Early-passage human keratinocytes or dermal fibroblasts were transduced by single exposure to MFG-lacZ retrovirus. Transduction efficiencies, as assessed by X-Gal staining of a representative culture, were greater than 80%. No change in the number of β-Gal-expressing cells was observed throughout the course of these studies. Nonselected pools of MFG-lacZ-transduced cells were incubated with several proinflammatory cytokines for 40 h at concentrations indicated in Fig. 1A. β-Gal activity in cell lysates of treated cultures was measured and compared to that of nontreated cultures. Of cells treated with the five cytokines tested, only IFN-γ-treated cells showed a significant reduction (>75%) in β-Gal activity compared to nontreated cells (Fig. 1A). Treatment of keratinocytes with

TNF-α also resulted in a slight reduction of β-Gal activity (~24%), but in TNF-α-treated fibroblasts, the enzyme activity did not significantly differ from that of nontreated cells. The effect of IFN-γ was evident at concentrations as low as 0.1 ng/ml; the maximal level, 5 ng/ml, required at least a 24-h incubation period to attain. Inhibition was not complete and did not go beyond 80 to 85% reduction in β-Gal activity even with higher levels of IFN-γ and longer incubation time (Fig. 1B and C).

The changes in transgene expression brought on by IFN-γ were not accompanied by detectable cytotoxic and/or cytostatic effects. For fibroblasts treated with IFN-γ for 40 h, the number of cells present and the amount of protein recovered were the same as for nontreated controls. For keratinocytes treated in the same way, there were 10% fewer cells recovered from treated cultures, whereas protein recovery was the same in treated and nontreated cultures (data not shown). As keratinocyte cultures are heterogeneous, with a mixture of small basal cells and large differentiated cells, we consider protein levels to be more indicative of the overall viability.

To determine whether the IFN-mediated inhibition of β-Gal activity was due to lower levels of transcript, the steady-state level of *lacZ* mRNA was analyzed. Total RNA was isolated from nontreated cultures or cultures treated with IFN-γ (5 ng/ml) for various periods of time (0 to 72 h). Levels of *lacZ* transcript in IFN-treated cells were compared with those of the nontreated cells by hybridization with a specific antisense RNA probe spanning *lacZ* coding sequences. An antisense probe for β-actin added simultaneously served as an internal control. As shown in Fig. 2, a significant decrease in the level of *lacZ* transcript was observed when either keratinocytes or fibroblasts were treated with IFN-γ (upper bands in Fig. 2A, lanes 3 to 7, and 2C, lane 3). No endogenous *lacZ* transcript was detected in normal fibroblasts (Fig. 3C) or keratinocytes (data not shown). IFN-γ did not affect β-actin mRNA levels (lower bands) even after 72 h of incubation, indicating that IFN-γ-mediated inhibition of *lacZ* expression was specific for LTR-driven *lacZ* transcript and was not related to cytotoxic effects of this cytokine on transduced cells.

The decrease in *lacZ* mRNA levels following exposure to IFN-γ was more rapid than the decrease in enzyme activity. Maximum inhibition of 70 to 80% in the levels of *lacZ* transcript (Fig. 2A, lane 5; Fig. 2C, lane 3) was observed after 16 h of incubation, compared to 24 h required for β-Gal activity. *lacZ* mRNA levels remained at reduced levels as long as the IFN-γ was present (incubation for up to 72 h was examined; Fig. 2A, lane 7). To determine whether the repression of LTR-directed expression was reversible, cells were treated with IFN-γ for 72 h and then washed and passed into fresh medium. Twenty-four or 48 h later, the levels of *lacZ* transcript were analyzed. As shown in Fig. 2B (lanes 2 and 3), upon removal of the cytokine, *lacZ* mRNA levels returned to normal, indicating that the inhibitory effects of IFN-γ were reversible. These results demonstrated a specific dose- and time-dependent repression of LTR-derived transgene expression in transduced cells as long as IFN-γ was present in the medium.

IFN-α also down regulates *lacZ* expression in transduced cells. Some of the antiviral and immunoregulatory effects of IFN-γ can be mimicked by other cytokines. For example, some proteins characteristically induced by IFN-γ are also induced by IFN-α or TNF-α (11, 15, 32). To evaluate the possible modulation of transgene expression by these cytokines, transduced keratinocytes were treated with TNF-α, IFN-α, or IFN-γ for 16 h, and the steady-state levels of *lacZ* transcript were analyzed as described above. A dose-response curve indicated that IFN-α mediated repression of β-Gal activity to the same extent as IFN-γ, with maximum inhibition at 250 IU of IFN-α per ml (data not shown). At this concentration, *lacZ* mRNA levels were reduced 75 to 80% over nontreated levels, comparable to levels observed following treatment with IFN-γ (Fig. 3, lanes 2 and 3). However, no synergistic effect was observed by a combination of IFNs (data not shown). Although incubation of keratinocytes with TNF-α (10 ng/ml) resulted in a slight reduction in β-Gal activity (Fig. 1A), no change in the level of *lacZ* transcript was observed (Fig. 3, lane 4). These results demonstrate that both IFN-α and IFN-γ suppressed LTR-driven *lacZ* expression and suggest that induction of a common polypeptide may have mediated their effects.

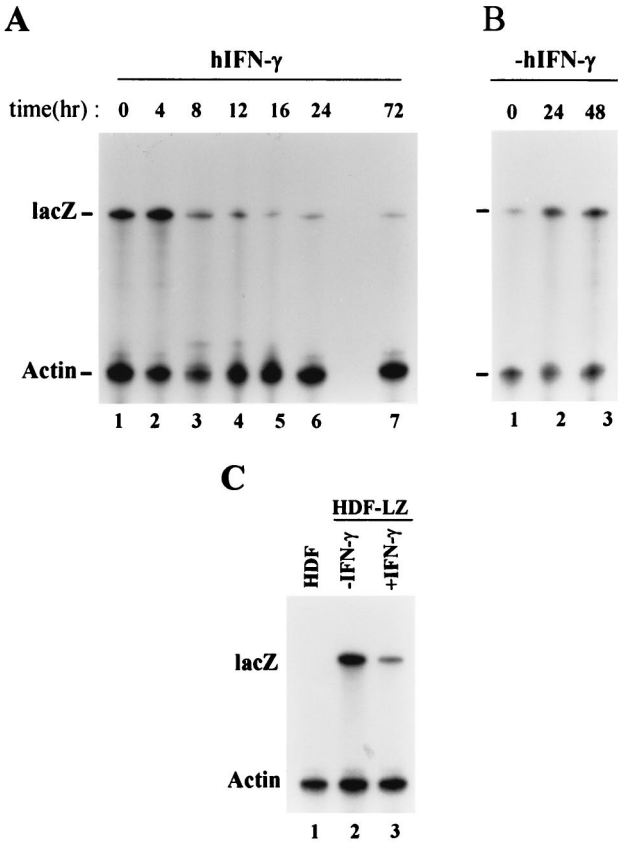

FIG. 2. Reduction in levels of steady-state *lacZ* mRNA in cells treated with IFN-γ. (A) Total RNA was isolated from transduced keratinocyte cultures nontreated (lane 1) or treated with human IFN-γ (hIFN-γ; 5 ng/ml) for the times indicated at the top (lanes 2 to 7). Steady-state mRNA levels for *lacZ* and β-actin were measured by hybridizing 10 μg of total RNA with specific antisense RNA probes (635 nt for *lacZ* and 270 nt for β-actin). RNase-protected fragments were analyzed by electrophoresis on a 5% denaturing polyacrylamide gel. (B) Transduced keratinocytes were incubated continuously over 72 h with IFN-γ (5 ng/ml), with one change of medium at 40 h. Cultures were harvested (lane 1) or passed into fresh medium without IFN for additional 24 h (lane 2) or 48 h (lane 3). RNA was isolated and analyzed as described in for panel A. (C) RNase protection analysis of RNA isolated from normal human dermal fibroblasts (HDF; lane 1) or MFG-lacZ-transduced human dermal fibroblasts (HDF-LZ) untreated (lane 2) or treated with IFN-γ (5 ng/ml; lanes 3), using the RNA probes described above.

The inhibitory effects of IFNs require de novo protein and RNA synthesis. The antiviral and immunoregulatory activities of IFNs are mediated by transcriptional activation of several genes. Transcription of some of these genes is enhanced within minutes and without the need for protein synthesis, while in several cases transcription of genes is enhanced after few hours during which new protein synthesis is required (10). Time

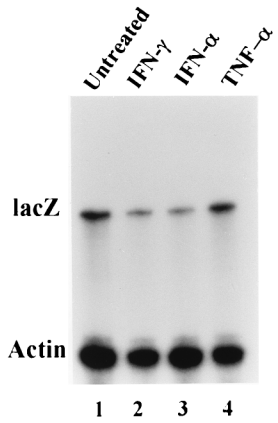

FIG. 3. Effects of IFN-α on LTR-directed transgene expression. Transduced keratinocytes were treated with IFN-γ (5 ng/ml; lane 2), IFN-α (250 IU/ml; lane 3), or TNF-α (10 ng/ml; lane 4) for 16 h. Total RNA was isolated from nontreated (lane 1) or treated cultures, and 10 ng of RNA was analyzed as described for Fig. 2.

course studies of keratinocytes indicated that IFN-mediated suppression of *lacZ* expression required 10 to 16 h of incubation with IFNs. To examine the requirement for protein and mRNA synthesis, cultures of transduced keratinocytes were pretreated with cycloheximide (20 μg/ml) or actinomycin D (1 μg/ml) for 30 min prior to addition of IFN-γ to block translation or transcription, respectively. Total RNA was isolated after 16 h of incubation with IFN-γ and analyzed by RNase protection assay. As shown in Fig. 4, although treatment of cultures with actinomycin D or cycloheximide alone resulted in a drop in *lacZ* transcript, additional treatment with IFN-γ failed to reduce these levels further (lanes 3 to 6). In contrast, there was a marked decrease in *lacZ* transcript levels in cultures treated with IFN-γ only (lanes 1 and 2). These results suggest that the IFN-mediated down regulation of LTR-directed gene expression is dependent on de novo protein and RNA synthesis.

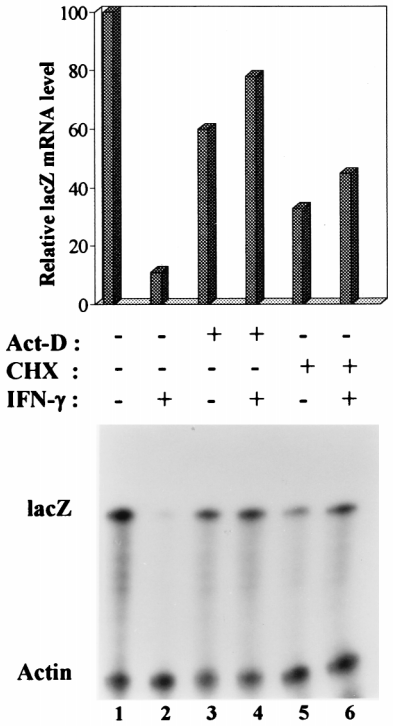

FIG. 4. Down modulation of *lacZ* mRNA by IFN-γ requires protein and RNA synthesis. MFG-lacZ-transduced keratinocytes were treated with the indicated agents for 16 h. When IFN-γ was used, it was added 30 min following the addition of other agents. Actinomycin D (Act-D) was used at 1 μg/ml (lanes 3 and 4), cycloheximide (CHX) was used at 20 μg/ml (lanes 5 and 6); IFN-γ was used at 5 ng/ml (lanes 2, 4, and 6). Total RNA was isolated and analyzed by RNase protection assay as described for Fig. 2. Protected fragments were cut from the gel, and the relative level of *lacZ* mRNA in each sample was quantified by a β-counter. Values within groups were normalized relative to the amount of β-actin mRNA and between groups to the dimethyl sulfoxide-treated control (lane 1).

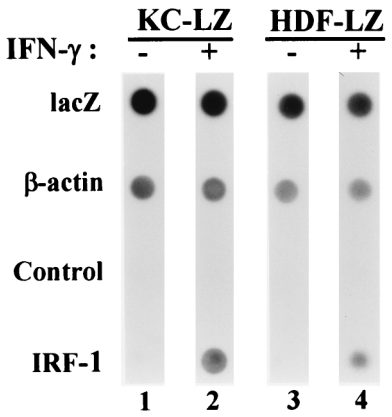

FIG. 5. Effects of IFNs on LTR transcriptional activity. Nuclear runoff assays were performed on nuclei isolated from 2×10^7 transduced keratinocytes (KC-LZ) or dermal fibroblasts (HDF-LZ) either untreated or treated with IFN-γ (5 ng/ml). ^{32}P-labeled runoff transcripts were hybridized to various DNAs bound to nylon membranes. IRF-1 is a gene known to be induced by IFN, and the appearance of IRF-1 transcripts shows that IFN-mediated signaling is activated. β-Actin was used as a control for normalization. The row labeled "Control" was spotted with DNA from an irrelevant plasmid.

Effect IFNs on the rate of transcription. To determine whether the decreased levels of steady-state *lacZ* mRNA observed following IFN treatment was the consequence of a decrease in the rate of transcription, nuclear run-off analysis was performed. Nuclei were isolated from cells treated with IFN-γ or nontreated cells, and transcripts were elongated in vitro as described in Materials and Methods. As shown in Fig. 5, the rate of *lacZ* transcription driven by LTR remained unchanged. As a control, transcription of IRF-1, an IFN-inducible gene, was induced as expected following incubation of cells with IFN-γ. Similar results were obtained in assays using IFN-α (data not shown). These results demonstrate that IFNs do not suppress LTR-mediated transcription and suggest that reduction in the levels of steady-state *lacZ* mRNA is regulated posttranscriptionally.

IFN-dependent modulation of *lacZ* transcript generated by an internal cellular promoter in a retroviral vector. The retroviral vectors used to this point contained a single promoter, the viral LTR. If the IFN-induced suppression is indeed mediated posttranscriptionally, transcripts originating from a second internal promoter but having the same 3′ end are likely to be affected similarly by IFN. To examine this possibility, the human involucrin promoter was inserted into the pBabe retroviral vector between the 5′ LTR and the *lacZ* gene to produce pBabe-inv-gal. (A similar retroviral vector construct without the involucrin promoter, pBabe-gal, was constructed and served as control.) The constructs and expected transcripts are depicted in Fig. 6A. The 3′ untranslated region including the poly(A) site is common between the transcripts initiated from

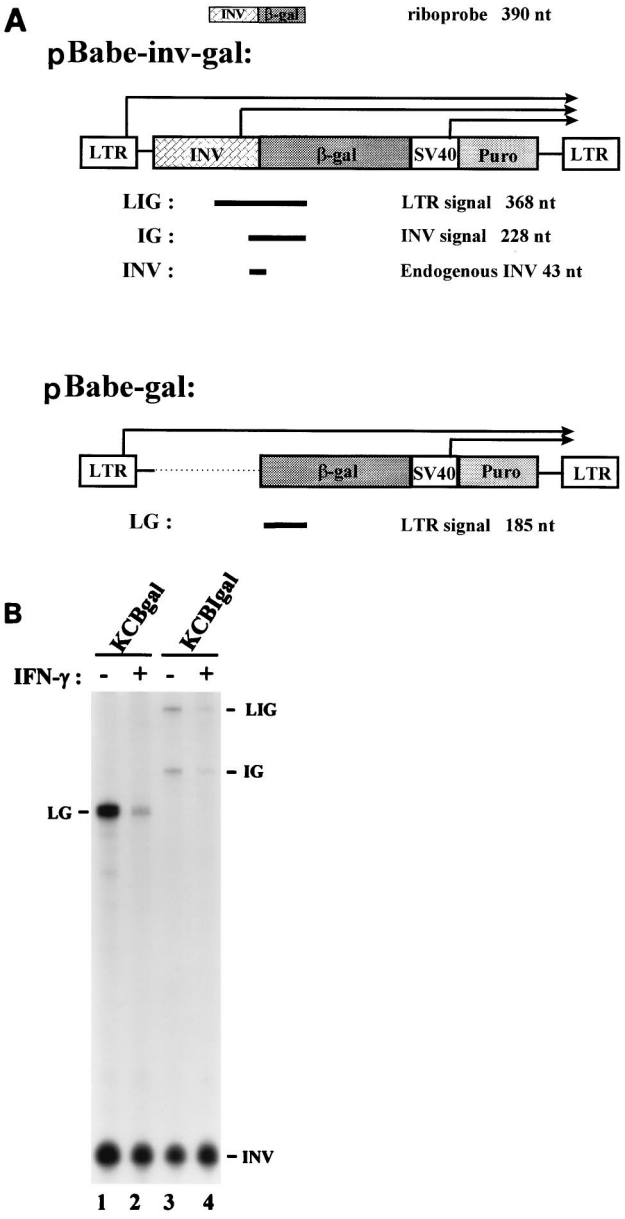

FIG. 6. Effects of IFN-γ on *lacZ* expression directed from an internal promoter in a retroviral vector. (A) Retroviral vector constructs in which *lacZ* expression is driven by the LTR (pBabe-gal) or an internal involucrin promoter (pBabe-inv-gal) are shown. The antisense RNA probe used for RNase protection assay is outlined at the top. The antisense RNA probe spans the initiation site for the involucrin promoter to differentiate LTR-initiated from involucrin-initiated transcripts. In cells transduced with pBabe-gal, a protected fragment (LG; 185 nt) is expected from transcripts generated by the vector, as well as a protected fragment (INV; 43 nt) from cellular involucrin mRNA. In cells transduced with pBabe-inv-gal, two protected fragments are expected, one from transcript derived from the LTR (LIG; 368 nt) and one initiated from the vector involucrin promoter (IG; 228 nt). A protected fragment (INV; 43 nt) from cellular involucrin mRNA is also expected. SV40, simian virus 40. (B) Keratinocytes transduced with pBabe-gal (KC-Bgal) or pBabe-inv-gal (KCBIgal) retrovirus were incubated with IFN-γ (5 ng/ml) for 16 h. Total RNA was isolated from IFN-treated or nontreated cultures, and 20 μg of total RNA was analyzed. Levels of transcripts initiated from each promoter were measured by specific hybridization to the antisense RNA probe diagrammed in panel A. The position of LTR-initiated (LIG), involucrin-initiated (IG), and endogenous involucrin (INV) transcripts are shown on the right; the position of LTR-initiated transcript in KCBgal (LG) is shown on the left. Protected fragments were analyzed on a 6% polyacrylamide-urea gel. The autoradiograph of the dried gel was exposed to X-ray film at −70 for 40 h. A representative experiment is shown.

the LTR and involucrin promoters. Keratinocytes were transduced with these retroviruses, selected with puromycin, and either not treated or treated with IFN-γ (5 ng/ml, 16 h). Total RNA was extracted, and the steady-state level of RNA initiated at each promoter was measured by RNase protection, using a riboprobe that spanned the involucrin transcriptional initiation site. This probe differentiated LTR-initiated from involucrin-initiated transcripts. Analysis of RNA from pBabe-gal-transduced keratinocytes demonstrated that in the presence of IFN-γ, the level of *lacZ* mRNA was reduced more than 75% compared to that of nontreated cells (Fig. 6B, lanes 1 and 2) confirming our previous results. In pBabe-inv-gal-transduced cells, the overall levels of *lacZ* transcript were lower than in pBabe-gal-transduced keratinocytes. This is likely the result of interaction between the two adjacent promoters, as has been reported previously by other investigators (7, 12). While the LTR promoter may have had an effect on transcription within involucrin promoter, the β-Gal expression by this vector remained keratinocyte specific (unpublished observation). Incubation of pBabe-inv-gal-transduced cells with IFN-γ resulted in a significant reduction in the levels of *lacZ* transcripts generated from both viral and involucrin promoters (lanes 3 and 4). The levels of endogenous involucrin mRNA in treated cells remained comparable to those in nontreated cells (lower bands in lanes 1 to 4), indicating the specificity of IFN-mediated suppression of transgene. These results demonstrate that IFN specifically modulated expression of the transgene directed by either LTR or an internal cellular promoter in the context of a retrovector and suggest that IFN-mediated suppression of expression is not promoter dependent.

DISCUSSION

Replication-defective MMLV-based retroviral vectors are the most popular method for gene transfer in ex vivo gene therapy protocols. Although the LTR promoter is active in tissue culture, it is poorly expressed in vivo for reasons that are not well understood (25, 39). We have used MMLV-based retroviral vectors expressing a reporter gene to study the effects of host-derived cytokines on transgene expression in cultured cells. Treatment of transduced human fibroblasts or keratinocytes with IFN-α and -γ resulted in a >75% reduction in both β-Gal activity and steady-state *lacZ* mRNA levels in a time- and dose-dependent manner. Maximal reduction in *lacZ* mRNA levels occurred prior to maximal reduction in β-Gal activity, suggesting that the primary event was a decrease in *lacZ* mRNA expression, not protein stability. The concentrations of IFNs that resulted in maximal suppression of β-Gal expression is known to maximally activate IFN-inducible factors (22). The modulatory effects of IFNs were shown to be specific to the retrovirus-expressed transgene, since the levels of cellular transcripts including β-actin and involucrin remained unaffected. Addition of IFN to keratinocytes at doses used in our experiments did not result in a significant change in growth and differentiation pattern of keratinocytes in submerged or organotypic cultures or that of the fibroblasts (unpublished observation and reference 20).

When transfection was used to introduce the viral vector constructs into keratinocytes instead of transduction, IFN-mediated inhibition of *lacZ* expression was not seen (data not shown), suggesting that integration of the transgene may be required. The site of provirus integration could not be a key factor in IFN-mediated down regulation of LTR-driven expression because polyclonal populations of transduced cells were used throughout these experiments, and similar results were obtained with either keratinocytes or fibroblasts and sev-

eral different MMLV-based vectors, including MFG and pBabe (see Results). Possibly transfected vector constructs did not exhibit IFN-induced inhibition of *lacZ* expression because of transfer of multiple copies of the plasmid DNA and possible titration of inhibitory factors. Consistent with this suggestion, we have noted that the inhibitory effect of IFN on *lacZ* expression is reduced when cells are transduced at titers higher than that reported in this study (data not shown). IFN-mediated suppression of transfected gene expression has been found when cytomegalovirus or simian virus 40 promoters were used to drive transgene expression. However, these effects were cell type dependent since they were not observed in some of the cell lines used (13, 19).

The fact that IFN-mediated down regulation of *lacZ* expression requires mRNA and protein synthesis and an incubation time of >8 h implies that suppression of transgene expression is among the secondary responses to IFNs and is likely mediated by synthesis of new protein intermediates. Furthermore, treatment of transduced cells with either IFN-α or IFN-γ resulted in inhibition of LTR-directed expression. As no synergy was noted for IFN-α and IFN-γ, it is likely that the effect of each cytokine was mediated by a common factor(s). Noteworthy, IFN's ability to elicit an antiviral state is due to transcriptional induction of quiescent genes and resultant production of new proteins (10).

The antiviral activity of IFNs is well documented and is based on activation of several mechanisms which may affect various phases of the virus growth cycle, including penetration, transcription, translation, virion assembly, and budding (27, 45). Although transduced cells are free of helper virus and no viral protein is made by the vector, the possibility of interaction of an IFN-induced factor with viral sequences left in the vector cannot be excluded. The down modulation of gene expression at transcriptional and posttranscriptional levels by IFNs has been reported. For example, inhibition of murine cytomegalovirus immediate-early gene transcription and *tat*-induced transactivation of human immunodeficiency virus LTR-driven expression of genes by IFN-γ have been demonstrated (13, 17). At the posttranscriptional level, IFN-γ has been shown to decrease stability of several transcripts, including c-*myc*, c-*fos*, and *cftr* (1, 8, 41). For reovirus-infected cells, a specific increase in the turnover rate of several viral transcripts has been reported (38). Moreover, inflammatory cytokines including IFN-γ and TNF-α have been shown to activate hepatitis B virus-infected hepatocytes to degrade viral RNA in a sequence-specific manner without affecting the rate of viral transcription (18, 50). Using nuclear runoff analysis to assess the effect of IFNs on the rate of transcription initiated from the LTR in transduced cells, we showed that suppression of *lacZ* mRNA levels was not due to transcriptional regulation of the LTR. The inability to detect changes in the rate of transcription was not due to low sensitivity of our assay since transcriptional induction of an IFN-inducible gene (IRF-1) was readily detected following IFN treatment (Fig. 5) (34). Therefore, the reduced level of transgene mRNA is due to regulation at the posttranscriptional level, most likely at the level of RNA stability. This conclusion is supported by the fact that IFN also reduced expression of a gene linked to involucrin promoter when that promoter was placed in an internal position in the retroviral vector. Promoter interference has been shown to reduce the ability of the adjacent promoter to act as an efficient promoter, and this interference could be relieved by suppression of upstream promoter (7, 30). The lower expression of *lacZ* initiated from both promoters in pBabe-inv-gal-transduced cells suggests interference between the two promoters (Fig. 6B). However, IFN treatment resulted in suppression of both LTR- and involucrin-initiated transcripts, which suggests that IFN-mediated suppression was not at the level of transcription. The only common viral sequences in the LTR and involucrin transcripts are sequences located at the 3′ ends, predominantly in the 3′ LTR. Preliminary studies in this laboratory suggest that the U3 region of the LTR mediates this IFN-induced suppression. The importance of 3′ untranslated region and polyadenylation signal in RNA stability and regulation of gene expression has recently been appreciated (23). In the design of retroviral vectors, these regions are among major viral sequences retained whether viral or internal promoters are used. Poor expression of retrovirus-delivered transgene has often been attributed to reduced expression of viral promoter in vivo; however, an internal cellular promoter does not always circumvent this problem, as expression remains less than that of the endogenous cellular promoter (30, 43, 52). Our data suggest the importance of posttranscriptional events in regulation of transgene expression, and we believe that modification of viral sequences involved in mRNA stability might be critical for achieving persistent and high-level transgene expression.

The stimulus for this study was to examine host factors that might alter expression of a retrovirus-transduced transgene in a gene therapy situation. Our discovery that IFNs suppress expression of both an LTR-regulated gene and a gene regulated by an internal cellular promoter not normally responsive to these cytokines has these important consequences: first, it indicates that host factors can and are likely to alter the results of gene therapy trials and should be considered in the design of such trials; second, it indicates that incorporation of a cellular promoter in an internal position in the vector may not insulate the transgene against unwanted modulating factors; and third, it suggests that further vector modifications should be directed at the 3′ end to reduce or eliminate changes in mRNA stability brought on by inflammatory cytokines.

ACKNOWLEDGMENTS

We thank Nancy Reich (SUNY at Stony Brook) for providing IRF-1 cDNA and human IFN-α, Russell Taichman (University of Michigan) for proinflammatory cytokines, and Richard Mulligan (Whitehead Institute) for the MFG-lacZ producer line. We also thank Li Ge and Robin Harrington for assistance in making the producer lines.

This research was supported by grants from NIH (RO1-DK49093 and R37-DEO4511). S. Ghazizadeh is a recipient of an NIH National Research Service Award.

REFERENCES

1. **Besancon, F., G. Przewlocki, I. Baro, A.-S. Hongre, D. Escande, and A. Edelman.** 1994. Interferon-gamma downregulates CFTR gene expression in epithelial cells. Am. J. Physiol. **267:**C1398–C1404.
2. **Carroll, J. M., K. M. Albers, J. A. Garlick, R. Harrington, and L. B. Taichman.** 1993. Tissue- and stratum-specific expression of the human involucrin promoter in transgenic mice. Proc. Natl. Acad. Sci. USA **90:**10270–10274.
3. **Challita, P.-M., and D. B. Kohn.** 1994. Lack of expression from a retroviral vector after transduction of murine hematopoietic stem cells is associated with methylation *in vivo*. Proc. Natl. Acad. Sci. USA **91:**2567–2571.
4. **Choate, K. A., D. A. Medalie, J. R. Morgan, and P. A. Khavari.** 1996. Corrective gene transfer in the human skin disorder lamellar ichthyosis. Nat. Med. **2:**1263–1267.
5. **Chomczynski, P., and N. Sacchi.** 1987. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
6. **Collins, T., L. A. Lapierre, W. Fiers, J. L. Strominger, and J. S. Pober.** 1986. Recombinant human tumor necrosis factor increases mRNA levels and surface expression of HLA-A,B antigens in vascular endothelial cells and dermal fibroblasts in vitro. Proc. Natl. Acad. Sci. USA **83:**446–450.
7. **Cullen, B. R., P. T. Lomedico, and G. Ju.** 1984. Transcriptional interference in avian retroviruses—implications for the promoter insertion model of leukaemogenesis. Nature **307:**241–245.
8. **Dani, C., N. Mechti, M. Peichaczyk, B. Lebleu, P. Jeanteur, and J. M. Blanchard.** 1985. Increased rate of degradation of c-myc mRNA in interfer-

on-treated Daudi cells. Proc. Natl. Acad. Sci. USA **82:**4896–4899.
9. **Danos, O., and R. C. Mulligan.** 1988. Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. Proc. Natl. Acad. Sci. USA **85:**6460–6464.
10. **Darnell, J. E., Jr., I. M. Kerr, and G. R. Stark.** 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science **264:**1415–1421.
11. **David, M.** 1995. Transcription factors in interferon signaling. Pharmacol. Ther. **65:**149–161.
12. **Emerman, M., and H. M. Temin.** 1984. Genes with promoters in retrovirus vectors can be independently suppressed by an epigenic mechanism. Cell **39:**459–467.
13. **Emilie, D., M. C. Maillot, J.-F. Nicolas, R. Fior, and P. Galanaud.** 1992. Antagonistic effect of interferon-gamma on tat-induced transactivation of HIV long terminal repeat. J. Biol. Chem. **267:**20565–20570.
14. **Fenjves, E. S., S.-N. Yao, K. Kurachi, and L. B. Taichman.** 1996. Analysis of loss of expression of a retrovirus-transduced gene in grafted human keratinocytes. J. Invest. Dermatol. **106:**576–578.
15. **Fujita, T., L. F. L. Reis, N. Watanabe, Y. Kimura, and T. Taniguchi.** 1989. Induction of the transcription factor IRF-1 and interferon-β mRNAs by cytokines and activators of the second-messenger pathways. Proc. Natl. Acad. Sci. USA **86:**9936–9940.
16. **Gerrard, A. J., D. L. Hudson, G. G. Brownlee, and F. M. Watt.** 1993. Towards gene therapy for haemophilia B using primary human keratinocytes. Nat. Genet. **3:**180–183.
17. **Gribaudo, G., S. Ravaglia, A. Caliendo, R. Cavallo, M. Gariglio, M. G. Martinotti, and S. Landolfo.** 1993. Interferons inhibit onset of murine cytomegalovirus immediate-early gene transcription. Virology **197:**303–311.
18. **Guidotti, L. G., T. Ishikawa, M. V. Hobbs, B. Matzke, R. Schreiber, and F. V. Chisari.** 1996. Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. Immunity **4:**25–36.
19. **Harms, J. S., and G. A. Splitter.** 1995. Interferon-gamma inhibits transgene expression driven by SV40 or CMV promoters but augments expression driven by the mammalian MHC I promoter. Hum. Gene Ther. **6:**1291–1297.
20. **Hertle, M. D., P. H. Jones, R. W. Groves, D. L. Hudson, and F. M. Watt.** 1995. Integrin expression by human epidermal keratinocytes can be modulated by interferon-gamma, transforming growth factor-beta, tumor necrosis factor-alpha, and culture on a dermal equivalent. J. Invest. Dermatol. **104:**260–265.
21. **Hoeben, R. C., A. A. Migchielsen, R. C. van der Jagt, H. van Ormondt, and A. J. van der Eb.** 1991. Inactivation of the Moloney murine leukemia virus long terminal repeat in murine fibroblast cell lines is associated with methylation and dependent on its chromosomal position. J. Virol. **65:**904–912.
22. **Horvath, C. M., and J. E. Darnell, Jr.** 1996. The antiviral state induced by alpha interferon and gamma interferon requires transcriptionally active Stat1 protein. J. Virol. **70:**647–650.
23. **Jackson, R. J.** 1993. Cytoplasmic regulation of mRNA function: the importance of the 3′ untranslated region. Cell **74:**9–14.
24. **Jaffee, E. M., G. Dranoff, L. K. Cohen, K. M. Hauda, S. Clift, F. F. Marshall, R. C. Mulligan, and D. M. Pardoll.** 1993. High efficiency gene transfer into primary human tumor explants without cell selection. Cancer Res. **53:**2221–2226.
25. **Kaleko, M., J. V. Garcia, and A. D. Miller.** 1991. Persistent gene expression after retroviral gene transfer into liver cells in vivo. Hum. Gene Ther. **2:**27–32.
26. **Kay, M. A., P. Baley, S. Rothenberg, F. Leland, L. Fleming, K. P. Parker, T.-J. Liu, M. Finegold, G. Darlington, W. Pokorny, et al.** 1992. Expression of human α1-antitrypsin in dogs after autologous transplantation of retroviral transduced hepatocytes. Proc. Natl. Acad. Sci. USA **89:**89–93.
27. **Kerr, I. M., and G. R. Stark.** 1992. The antiviral effects of the interferons and their inhibition. J. Interferon Res. **12:**237–240.
28. **Markowitz, D., S. Goff, and A. Bank.** 1988. Construction and use of a safe and efficient amphotropic packaging cell line. Virology **167:**400–406.
29. **Marzluff, W. F., and R. C. C. Huang.** 1984. Transcription of RNA in isolated nuclei, p. 89–129. *In* B. D. Hames and S. J. Higgins (ed.), Transcription and translation: a practical approach. IRL Press, Oxford, England.
30. **McCune, S. L., and T. M. Townes.** 1994. Retroviral vector sequences inhibit human beta-globin gene expression in transgenic mice. Nucleic Acids Res. **22:**4477–4481.
31. **Melton, D. A., P. A. Krieg, R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes for plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12:**7035–7056.
32. **Mestan, J., W. Digel, S. Mittnacht, H. Hillen, D. Blohm, A. Moller, H. Jacobsen, and H. Kirchner.** 1986. Antiviral effects of recombinant tumour necrosis factor *in vitro*. Nature **323:**816–819.
33. **Miller, A. D.** 1992. Retroviral vectors. Curr. Top. Microbiol. Immunol. **158:**3–24.
34. **Miyamoto, M., T. Fujita, Y. Kimura, M. Maruyama, H. Harada, Y. Sudo, T. Miyata, and T. Taniguchi.** 1988. Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-beta gene regulatory elements. Cell **54:**903–913.
35. **Morgenstern, J. P., and H. Land.** 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res. **18:**3587–3596.
36. **Ng, R. L. H., B. Woodward, S. Bevan, C. Green, and R. Martin.** 1997. Retroviral marking identifies grafted autologous keratinocytes in porcine wounds receiving cultured epithelium. J. Invest. Dermatol. **108:**457–462.
37. **NIH Recombinant DNA Advisory Committee.** 1996. Human gene therapy clinical protocols. Hum. Gene Ther. **7:**565–568.
38. **Nilsen, T. W., P. A. Maroney, and C. Baglioni.** 1982. Synthesis of (2′-5′)oligoadenylate and activation of an endoribonuclease in interferon-treated HeLa cells infected with reovirus. J. Virol. **42:**1039–1045.
39. **Palmer, T. D., G. L. Rosman, W. R. Osborne, and A. D. Miller.** 1991. Genetically modified skin fibroblasts persist long after transplantation but gradually inactivate introduced genes. Proc. Natl. Acad. Sci. USA **88:**1330–1334.
40. **Palmer, T. D., A. R. Thompson, and A. D. Miller.** 1989. Production of human factor IX in animals by genetically modified skin fibroblasts: potential therapy for hemophilia B. Blood **73:**438–445.
41. **Radzioch, D., and L. Varesio.** 1991. c-*fos* mRNA expression in macrophages is downregulated by interferon-γ at the posttranscriptional level. Mol. Cell. Biol. **11:**2718–2722.
42. **Rheinwald, J. G., and H. Green.** 1975. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinocyte colonies from single cells. Cell **6:**331–344.
43. **Richards, C. A., and B. E. Huber.** 1993. Generation of a transgenic model for retrovirus-mediated gene therapy for hepatocellular carcinoma is thwarted by the lack of transgene expression. Hum. Gene Ther. **4:**143–50.
44. **Riviere, I., K. Brose, and R. C. Mulligan.** 1995. Effects of retroviral vector design on expression of human adenosine deaminase in murine bone marrow transplant recipients engrafted with genetically modified cells. Proc. Natl. Acad. Sci. USA **92:**6733–6737.
45. **Samuel, C. E.** 1991. Antiviral actions of interferon. Interferon-regulated cellular proteins and their surprisingly selective antiviral activities. Virology **183:**1–11.
46. **Scharfmann, R., J. H. Alexrod, and I. M. Verma.** 1991. Long-term *in vivo* expression of retrovirus-mediated gene transfer in mouse fibroblast implants. Proc. Natl. Acad. Sci. USA **88:**4626–4630.
47. **Stead, R. B., W. W. Kwok, R. Storb, and A. D. Miller.** 1988. Canine model for gene therapy: inefficient gene expression in dogs reconstituted with autologous marrow infected with retroviral vectors. Blood **71:**742–747.
48. **Stockschlader, M. A., F. G. Schuening, T. C. Graham, and R. Storb.** 1994. Transplantation of retrovirus-transduced canine keratinocytes expressing the β-galactosidase gene. Gene Ther. **1:**317–322.
49. **Strauss, M.** 1994. Liver-directed gene therapy: prospects and problems. Gene Ther. **1:**156–164.
50. **Tsui, L. V., L. G. Guidotti, T. Ishikawa, and F. V. Chisari.** 1995. Posttranscriptional clearance of hepatitis B virus RNA by cytotoxic T lymphocyte-activated hepatocytes. Proc. Natl. Acad. Sci. USA **92:**12398–12402.
51. **Wu, Y.-J., L. M. Parker, N. E. Binder, M. A. Beckett, J. H. Sinard, C. T. Griffiths, and J. G. Rheinwald.** 1982. The mesothelial keratins: a new family of cytoskeletal proteins identified in cultured mesothelial cells nonkeratinizing epithelia. Cell **31:**693–703.
52. **Yee, J. K., J. C. Moores, D. J. Jolly, J. A. Wolff, J. G. Respess, and T. Friedmann.** 1987. Gene expression from transcriptionally disabled retroviral vectors. Proc. Natl. Acad. Sci. USA **84:**5197–5201.