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In Vivo Expression of an Interferon-α Gene by Intramuscular Injection of Naked DNA

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ABSTRACT

Acid-stable type I interferons belong to a multigene family. The biologic relevance of each subtype *in vivo* remains unknown. We have developed an experimental model in which muscles were transfected *in situ* with naked DNA plasmids encoding an IFN transgene to assess the roles of individual IFN subtypes *in vivo*. Murine IFN-α 9 gene was subcloned into several mammalian expression vectors. Adult C57BL/6 mice were injected bilaterally in regenerating tibialis anterior muscles with naked DNA 5 days after muscle injury to enhance DNA uptake and expression. In the muscles of mice given the IFN-α9 plasmid constructs, acid-stable IFNs were detected by bioassay using reduction in cytopathic effect of encephalomyocarditis virus-infected L929 cells. In these same muscles, IFN-α9 transcripts were identified by RT-PCR, indicating that transcription had occurred. Acid-stable IFNs were detected from days 7 to 28 post-DNA inoculation. Furthermore, these proteins were found in the sera of DNA-inoculated mice. Control groups of mice given the blank expression vectors did not produce detectable IFNs in muscle or sera as determined by bioassay, nor were transcripts detected by RT-PCR. This approach now allows investigation of the effector function of individual subtypes in various murine disease models.

INTRODUCTION

THE TYPE I INTERFERON (IFN) GENES belong to a multigene family located together on chromosome 9,[1] with over 15 α genes and one β gene expressed in humans.[2] Similarly, there are multiple type I IFN genes on chromosome 4[3] in the mouse, with over 10 α and 1 β subtype expressed.[4,5] Collectively, the IFNs have diverse biologic functions, including antiviral,[6,7] antiproliferative,[8] and immunomodulatory activities[9] mediated via signaling transduction pathways.[10] Although evidence from *in vitro* studies suggests differences in the biologic effects of the subtypes,[2,11–13] the significance of these *in vivo* remains obscure. Increased knowledge of the activities of the individual subtypes *in vivo* will enhance the use of such subtypes for improved clinical therapy over the current use of either a mixture of IFN subspecies or a single recombinant IFN subtype.

One approach to the expression of an individual IFN subtype *in vivo* was made using transgenic mice.[14–17] Transgenic mice were created with either murine *IFNA1* or murine *IFNB* under the control of the metallothionein-I promoter and unexpectedly expressed the gene only in the testes (IFN-α and IFN-β) and liver (IFN-β) on induction with cadmium. IFN was detected only in the sera of the IFN-β transgenic animals. However, both transgenic lines showed inhibition of spermatogenesis, resulting in sterility of male mice. No further experimental work with these animals has been reported. We have also unsuccessfully attempted to produce transgenic mice expressing either the murine IFN-α1 or IFN-α4 gene under the control of a mutated (nonleaky) metallothionein-IIA promoter (W.S. Yeow and M.W. Beilharz, unpublished observations). It is likely that even low levels of expression of the IFN transgene in the developing ova are conditionally lethal during embryogenesis.

The advent of naked DNA transfer technology provided an alternative approach to the manipulation of gene expression *in vivo*.[18] Regenerating muscle has been reported to have special ability for expression of intramuscularly inoculated plasmid-containing transgenes.[19,20] Immunization with plasmids expressing viral genes has induced protective immune responses, and these are being developed for improved vaccines.[21,22] The persistence of the plasmid allows ongoing expression of the transgene, and this finding has been pursued for gene therapy.[23]

In this article, we describe the delivery and expression of an individual murine IFN-α subtype, *IFNA9*, in mouse skeletal tib-

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ialis anterior (TA) muscle. Dot blot analysis showed that the transcript was present in the DNA-inoculated muscle. IFN levels were found in both muscle homogenate and sera of inoculated animals for up to 1 month post-DNA injection. We have compared expression levels of IFN driven by the human cytomegalovirus (HCMV) immediate early (IE) enhancer/promoter with that of the human β-actin promoter. In addition, we have compared different techniques of inducing muscle necrosis and regeneration on the expression of the transgene. The levels of expression obtained were comparable to those reported for the few IFN-α transgenic mice made, and the length of expression is at least 4 weeks. This technique can now be applied to other murine IFN subtypes and will allow a systematic analysis of the role of each subtype in experimental disease models.

MATERIALS AND METHODS

Mice

C57BL/6 (B6) female mice were purchased from Animal Resources Centre, (Murdoch, Western Australia) and were inoculated at 6–7 weeks of age.

Plasmid constructs

Plasmids were constructed with the 1.8 kb *Hind*III *MuIFNA9* gene cassette obtained from the SV40-pBR322 construct.[24] The murine gene was full length and included the upstream signal sequence (572 bp) (Fig. 1A). The *Hind*III fragment was subcloned into the pHβ.APr-1-neo expression vector at the *Hind*III site (Fig. 1B). This plasmid construct expressed biologically active IFN following transient transfections of L929 cells *in vitro*. The *Sal*I and *BamH*I digested pHβ.APr-1-neo.*IFNA9* construct released the *IFNA9* cassette, which was then subcloned into the pkCMVint expression vector kindly donated by VICAL (San Diego, CA) at the *Sal*I and *BamH*I sites (Fig. 1C).

Bulk plasmids were prepared from transformed *Escherichia coli* using standard DNA extraction procedures with LiCl precipitation. DNA integrity was checked by agarose gel electrophoresis, and concentrations were determined by spectrophotometric analysis.

Muscle regeneration

To induce muscle regeneration by crush injury,[25] mice were anesthetized with 2.5% avertin, fur was removed from the hind legs, and TA muscles were exposed by surgical incision of the skin. The TA muscles were sheared away from the underlying bones with forceps and crushed in three equally spaced locations along the muscles for 3 sec using artery forceps. The incisions were sutured, and the mice were allowed to recover for 5 days before inoculation with the DNA constructs.

Alternatively, to induce muscle regeneration by bupivacaine, anesthetized mice were injected bilaterally into the TA muscles

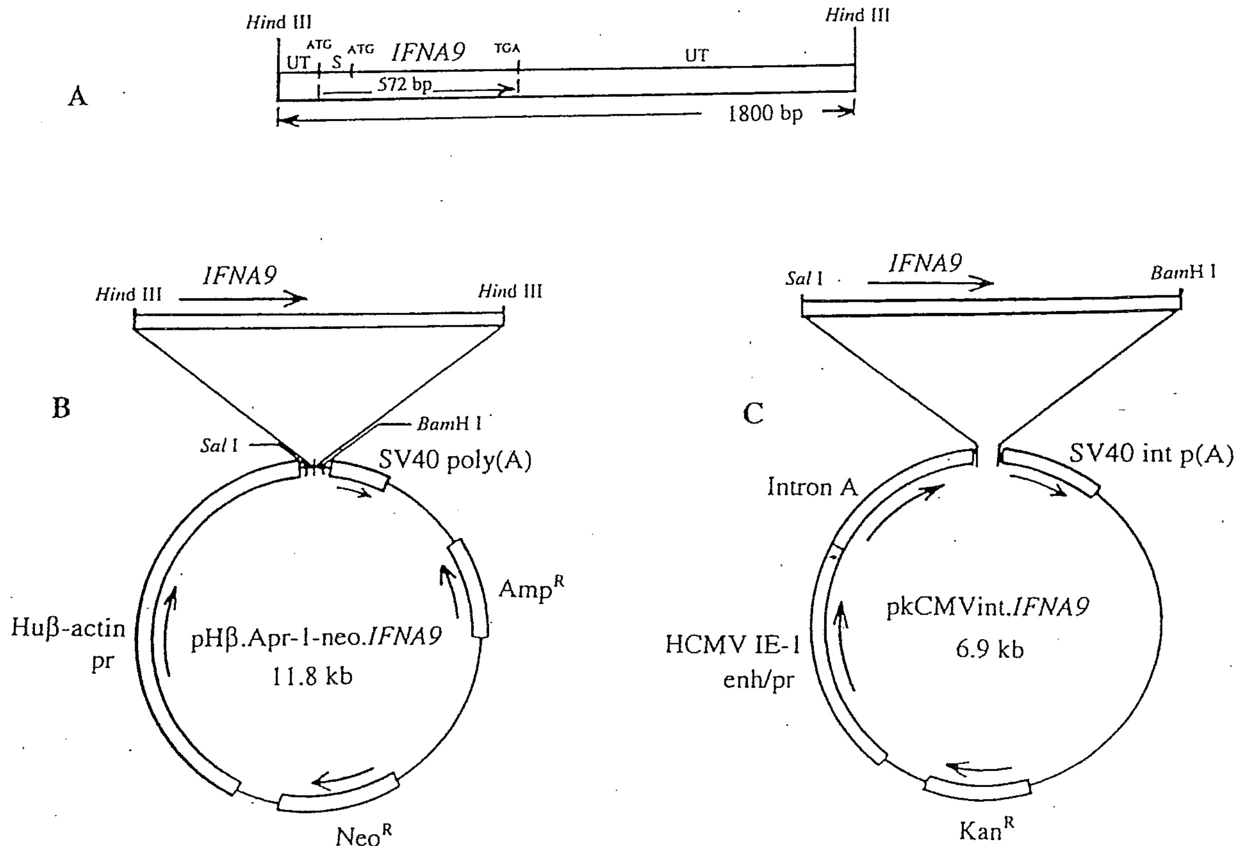

FIG. 1. Plasmid maps showing the (A) *IFNA9* gene cassette[24] subcloned into the mammalian expression vectors, (B) pHβ-APr-1-neo and (C) pkCMVint (see Materials and Methods for details of cloning).

with 50 μl of 0.5% bupivacaine 5 days before inoculation of the DNA constructs.

Injection of DNA constructs

Anesthetized mice were inoculated with 200 μg of DNA plasmids in a 50 μl volume of saline bilaterally in the TA muscles by inserting the needle longitudinally into the muscle. Similarly, control groups of mice were given saline without DNA or the blank expression vectors.

IFN bioassay

The TA muscles and blood were collected from the mice at days 7, 14, 21, and 28 following inoculation. Muscle homogenates (20% in saline) and sera were treated at pH 2 for 1 h at −20°C and then neutralized to pH 7. The supernatants containing acid-stable proteins were clarified by centrifugation at 8000*g* for 15 min, followed by a further high-speed centrifugation at 22,000*g* for 15 min at 4°C before titration in the IFN bioassay.

IFN titers were determined in an *in vitro* bioassay, and end point titers were defined as the reciprocal dilution giving a 50% reduction in cytopathic effect from encephalomyocarditis virus (EMCV) infection of L929 cells. The standard murine IFN-α/β (Lee Biomolecular, 1000 IU/ml) was also titrated in the bioassay as a control.

Dot blot analysis

An RT-PCR analysis of the extracted RNA was performed for identification of the type I IFN subtypes as described previously,[26,27] with modifications. Briefly, total cytoplasmic RNA isolated with RNAzol™ B (Biotecz, Houston, TX) from DNA-inoculated TA muscles was treated with DNase (Promega, Madison, WI). Total mRNA was reverse transcribed in the Gene-Amp system (Perkin Elmer, Branchburg, NJ) using AMV RT (Promega) and random hexamers (Promega). Amplification of all *IFNA* subtypes (308 bp fragment) was produced using AmpliTaq (Perkin Elmer) and the primer pair 5′-TCTCTCCTGCCTGAAGGAC-3′ and 5′-ACACAGTGATCCTGTGGAA-3′. Identification of the individual *MuIFNA* subtypes in the PCR products was made by hybridization to [γ-^{32}P]ATP-labeled oligonucleotides specific for *IFNA1* subtype (5′-ATTTCCCCTGACCCAGGAAGAT-3′), *IFNA4* subtype (5′-CCTGTGTGATGCAGGAACCTCC-3′, and *IFNA9* subtype (5′-GCTGGTCGGGATGAAGGAACTG-3′). Hybridization temperatures were 60°C for *MuIFNA9* and 65°C for *MuIFNA1* and *MuIFNA4*.

RESULTS

Comparison of IFNA9 *expression from β-actin and CMV promoter-driven plasmids*

Mice were injected with 200 μg of plasmid constructs carrying the full-length gene for murine type I *IFNA9* with either the human β-actin promoter (pHβ.APr-1-neo. *IFNA9*) (Fig. 1B) or the HCMV immediate early enhancer/promoter (pkCMVint.*IFNA9*) (Fig. 1C). Control groups of mice received the blank expression vectors, pHβ.APr-1-neo and pkCMVint or saline only. Regenerating TA muscles were induced by crush injury 5 days before DNA injection. The level of acid-stable IFN expression in the DNA-inoculated muscle is shown in Table 1. At both day 7 and day 14 post-DNA inoculation, type I IFN bioactivity was not detectable in the muscles of mice injected with either the blank control plasmids or saline. However, crush-injured muscles were able to take up and express the IFN-containing plasmids. It can be seen that at day 7, the pkCMVint.*IFNA9* plasmid produced greater titers (8-fold higher) of IFN than those induced by the pHβ.APr-1-neo.*IFNA9* plasmid, as determined by protection of L929 cells from cytopathic effects of EMCV infection *in vitro*. At day 14, similar levels of IFN were detected in the muscle of both groups of mice, although the levels in pkCMVint.*IFNA9*-inoculated muscles were slightly higher than those observed for pHβ.APr-1-neo.*IFNA9*-inoculated muscles. The number of mice positive for IFN expression in a group varied between 43% and 86%, suggesting that the technique of DNA transfer and expression is suboptimal. Because the CMV promoter was superior to the β-actin promoter, in further experiments we used the pkCMVint.*IFNA9* plasmid containing the CMV promoter.

TABLE 1. HIGHER LEVELS OF IFN PROTEIN WERE FOUND IN MUSCLES OF MICE INJECTED WITH PKCMVINT.*IFNA9* VERSUS PHβ.APR-1-NEO.*IFNA9*[a]

	Acid-stable IFN titers (IU/mouse)[b]	
Plasmid	*Day 7*	*Day 14*
pHβ.APr-1-neo.*IFNA9*	8.1 ± 2.1 (4/7)	37.5 ± 0.0 (3.7)
pHβ.APr-1-neo	≤1.7 ± 0.0 (0/3)	≤1.7 ± 0.0 (0/3)
pkCMVint.*IFNA9*	63.4 ± 24.0 (3/5)	48.0 ± 15.6 (6/7)
pkCMVint	≤1.7[c]	ND
Saline	≤1.7 ± 0.0 (0/2)	≤1.7 ± 0.0 (0/2)

[a]Plasmid DNA (200 μg in saline) was injected bilaterally into regenerating TA muscles of adult B6 mice that were crush injured 5 days earlier.

[b]Acid-stable IFN protein titers determined by bioassay using individual mouse muscle homogenates. The mean ± SEM is shown for the number of positive mice in the group given in parentheses.

[c]Titers determined for pooled muscle samples from 5 mice.

Identity of IFN transcript

To identify the transgene mRNA in the pkCMVint.*IFNA9*-inoculated muscles, we extracted total muscle RNA at day 7 and performed an RT-PCR-based identification analysis using probes specific for several type I IFN-α subtypes. The PCR primers are conserved for all the type I IFN-α subtypes. Since the murine type I IFN genes do not contain introns, it is important to differentiate the input mRNA from genomic DNA as template material for the PCR. Thus, all RNA extracts from the muscles are DNase treated. As a control for determining the level of residual DNA after DNase treatment of the isolated RNA extract, a direct PCR was done on the RNA sample from mice inoculated with pkCMVint.*IFNA9*. No detectable DNA product for type I IFN-α was amplified in the direct PCR, and it could not hybridize with either *IFNA9* or *IFNA1* by dot blotting (Fig. 2A*,B*). However, when the RNA extract was reverse transcribed and amplified by PCR, the product could by hybridized with the *IFNA9* probe (Fig. 2A, Table 2) but not the *IFNA1* probe (Fig. 2B, Table 2) or *IFNA4* probe (Table 2). These results indicate the presence of a specific *IFNA9* transcript from the inoculated DNA plasmid.

Similarly, muscle that was induced to regenerate by treatment with bupivacaine also demonstrated *IFNA9* transcript in the pkCMVint.*IFNA9*-inoculated muscle (Table 2). In addition, mature uninjured muscle that was not allowed to undergo regeneration before DNA injection also showed a specific hybridization signal with the *IFNA9* probe, whereas mice given the blank vector pkCMVint did not produce IFN transcripts in the DNA-inoculated muscles, as detected by RT-PCR-based identification analysis (Fig. 2, Table 2).

The hybridization signal for *IFNA9* from crush-injured muscle was stronger than that observed for bupivacaine-treated or normal muscle (Table 2). This was in agreement with the protein titers in the bioassay, which showed approximately a 3-fold to 5-fold increase in titer for the regenerating crush-injured muscle compared with the uninjured or bupivacaine-injured muscle in the expression of IFN at 7 days post-pkCMVint.*IFNA9* inoculation (18.8 IU/mouse for uninjured muscle) (Tables 1 and 3).

Persistence of IFN expression in muscle

We next examined the kinetics of IFN expression in the pkCMVint.*IFNA9*-inoculated animals. Mice were injected with

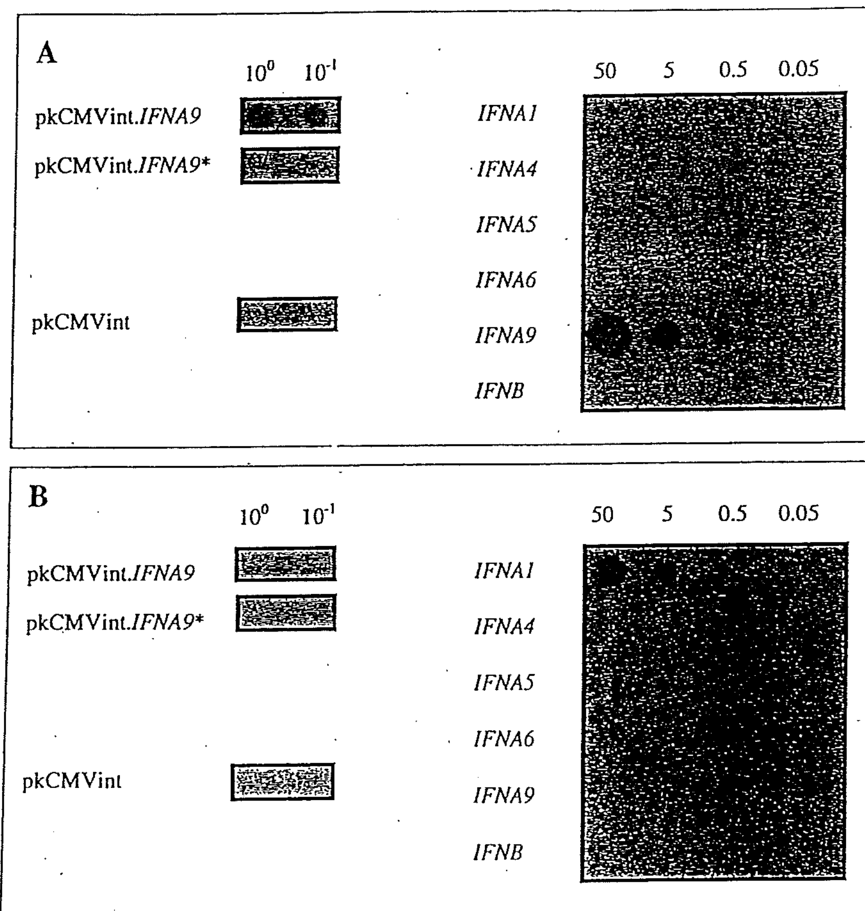

FIG. 2. The *IFNA9* transcript is identified in muscle extracts following intramuscular DNA inoculation. Dot blot analysis using IFN-specific probes for *IFNA9* shown in A and for *IFNA1* shown in B. At left, RT-PCR products from pooled muscle RNA extracts taken at day 7 post-DNA injection are shown as undiluted and 1:10 dilutions. *Direct PCR of the RNA without RT to assess for residual DNA in the DNase-treated RNA extracts. At right, IFN gene standards are titrated from 50 to 0.05 ng DNA for a given Mu-IFN subtype.

TABLE 2. PRESENCE OF *IFNA9* TRANSCRIPT AT DAY 7 POSTINJECTION OF PKCMVINT.*IFNA9* IN TA MUSCLE[a]

Injured muscle[b]	*Plasmid*	*MuIFNA1*[c]	*MuIFNA4*	*MuIFNA9*
Crush	pkCMVint.*IFNA9*	–	–	++
Bupivaccaine	pkCMVint.*IFNA9*	–	ND	+
No injury	pkCMVint.*IFNA9*	–	–	+
Crush	pkCMVint	–	–	–
Bupivaccaine	pkCMVint	–	ND	–

[a]Mice were inoculated with 200 μg plasmid bilaterally in the TA muscles, and mRNAs obtained in the muscles taken at day 7 post-DNA inoculation were standardized for mouse β-actin by RT-PCR and subjected to dot blot analysis.

[b]Necrosis and regeneration of muscle were induced either by crush injury or bupivacaine treatment 5 days before DNA injection.

[c]The cDNA PCR products were dot blotted and hybridized with specific probes for *IFNA1*, *IFNA4*, and *IFNA9*. +++, >200 ng; ++, >50 ng; +, 5 ng; –, ≤5 ng.

TABLE 3. IFN EXPRESSION IN MUSCLE PERSISTS UP TO DAY 28 FOLLOWING A SINGLE DNA INOCULATION[a]

Days after DNA inoculation	*Acid-stable IFN titers (IU/mouse)*[b]
7	13.6 (n = 6)
14	27.2 (n = 6)
21	37.5 (n = 6)
28	75 (n = 6)

[a]Mice were inoculated with 200 μg pkCMVint.*IFNA9* plasmid bilaterally in regenerating TA muscles treated with bupivacaine 5 days earlier.

[b]Acid-stable IFN protein titers determined by bioassay using pooled TA homogenates.

a single dose of DNA bilaterally in regenerating TA muscles, and IFN protein titers were determined from muscle homogenate pools of six mice at days 7, 14, 21, and 28 post-DNA inoculation. It can be seen in Table 3 that IFN was detected at each time point and the titer increased over the 4 weeks following DNA injection. The average titers from pooled muscle homogenates (Table 3) may include some mice with undetectable IFN, and, thus, there would be a lower estimate of the actual titer obtained from only the positive expressing mice (Table 1). Furthermore, IFN was detected in crush-injured and DNA-injected muscle as late as day 57 (150 IU/mouse). Again, mice injected with blank vector, pkCMVint, did not produce detectable IFN titers in the DNA-inoculated muscles (data not shown).

Circulating IFN protein levels in serum

Mice injected with pkCMVint.*IFNA9* into either crush-injured muscle, bupivacaine-injured muscle, or normal mature muscle were monitored for circulating levels of IFN in the sera. IFN titers were detected in groups of mice with regenerating muscle induced by crush injury but were not detected in groups of mice with either regenerating muscle induced by bupivacaine treatment or normal mature muscle (Table 4). Although the

TABLE 4. SERUM IFN PROTEIN IS FOUND IN MICE AFTER INJECTION OF PKCMVINT.*IFNA9* IN CRUSH-INJURED MUSCLE

	Acid-stable IFN titers in sera (IU/ml)[a]		
Days after DNA inoculation	*Crush injury*[b]	*Bupivacaine injury*[c]	*Normal muscle*
7	22.0 ± 6.0 (3/5)	ND	≤3.9
14	36.9 ± 25.9 (2/7)	≤3.9 ± 0.0 (0/5)	≤3.9

[a]Acid-stable IFN protein titers determined by bioassay using individual or pooled mouse muscle homogenates. Mice were inoculated with 200 μg pkCMVint.*IFNA9* plasmid bilaterally in TA muscles. The mean ± SEM is shown for the number of positive mice in the group given in parentheses.

[b]Regenerating muscle was induced by crush injury 5 days before DNA inoculation.

[c]Regenerating muscle was induced by bupivacaine injury 5 days before DNA inoculation.

number of mice positive for serum IFN was low, the titer showed a moderate increase from day 7 to day 14 following DNA injection.

DISCUSSION

IFN gene transfer into muscle

In our study, mice injected with the *IFNA9* gene constructs were capable of transcription and translation of the transgene. A comparison of DNA expression from two vectors driven by either human β-actin or HCMV immediate early promoter showed that the viral promoter was stronger and, thus, pkCMVint is a better expression vector for transfer of IFN into muscle. This vector also contains the HCMV intron A, which shares homology to an internal regulatory element of the muscle gene, troponin I.[28] Transgenic studies have suggested that this viral sequence directs gene expression primarily in muscle tissue.[29] An important control finding was that the delivery of DNA itself into the muscle and the injury procedure did not induce a detectable endogenous IFN response. IFN was detectable only when the gene was present in the DNA construct.

In addition, regenerating crush-injured muscle showed improved DNA expression over normal mature muscle and also over bupivacaine-injured muscle. Previous studies have found greater DNA expression using regenerating muscle than mature uninjured muscle.[19,20,30] It is likely that regenerating muscle allows for increased plasmid uptake within the muscle and increased access of the DNA to the sarcolemma. Bupivacaine was used as an alternative to crush injury for the induction of muscle regeneration. It is myotoxic and causes increased membrane permeability to calcium ions, which induces necrosis and hypercontraction. Following both procedures, the muscle regenerates rapidly, with myotube formation after 3 days and normal muscle architecture fully restored by 21 days.[31] Although the IFN protein titers in the DNA-inoculated regenerating muscles were detected using both types of muscle injury, an unexpected finding was that circulating IFN was only evident in the sera of DNA-inoculated mice that had regenerating muscles induced by crush injury and not by bupivacaine. Perhaps the damage to the TA muscles was greater with crush injury such that the physical barrier of the muscle bundle fascia was broken down to allow leakage of the expressed IFN into the bloodstream.

Gene transfer into regenerating muscle was less than 100% efficient, suggesting that the DNA was not productively transferred in all the mice. The injection technique associated with DNA delivery to the TA muscles, including the diffusion of the DNA within the muscle, cellular uptake of the DNA into the nucleus, leakage of the DNA inoculum from the injection site, and DNA degradation postinoculation, may contribute to the suboptimal process of naked DNA transfer.

However, there are factors other than efficiency that make naked DNA the chosen gene transfer approach. The relatively simple procedure of naked DNA transfer for IFN genes contrasts with the expensive and technically demanding production of transgenic mice for *in vivo* analysis of specific IFN gene function. Although the IFN expression levels were comparable for both DNA-inoculated animals and transgenic animals, the failure of the type I IFN transgenic mice to thrive and thus produce a colony for experimental purposes makes this an unviable approach. On the other hand, transgene DNA has been shown to persist and express for up to the lifetime of the murine host without undesirable effects.[32] Long-term stability of the plasmid in adult muscle provides opportunities for experimental research. In our study, we demonstrated the presence of the transcript from the introduced *IFNA9* gene as early as 7 days post-DNA transfer, with evidence of IFN expression up to 57 days.

Potential for IFN subtype analysis

The ability of mice to express a particular IFN subtype using the naked DNA gene transfer approach is reported here for the first time. The question of whether or not certain individual subtypes are more effective *in vivo* than others has not been fully addressed despite the fact that the human[33] and mouse subtype activities are known to vary significantly *in vitro*. For example, Mu-IFN-α4 has 10-fold higher *in vitro* antiproliferative activities than the Mu-IFN-α1 subtype.[13] In addition, Mu-IFN-α4 shows 2-fold greater *in vitro* antiviral activities than Mu-IFN-α1.[34]

Exogenous IFNs administered to humans and animals have produced a spectrum of efficacies ranging from good to poor.[35–37] However, the individual role of the IFN subtypes is unclear in these studies, as they often were conducted with undefined mixtures of subtypes derived from viral induction of cells or with only a single pure recombinant subtype. The surprisingly simple technique of gene transfer via intramuscular injection of naked DNA allows further investigation of the relative *in vivo* efficacies of the functional capabilities of each type I IFN subtype in animal models. We are currently systematically analyzing antitumor and antiviral actions of the murine IFNs in established animal models of disease.

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