Intervirology

Intervirology 2000;43:258–272

Plasmid DNA Vaccines: Investigation of Integration into Host Cellular DNA following Intramuscular Injection in Mice

Brian J. Ledwith Sujata Manam Philip J. Troilo Amy B. Barnum
Cindy J. Pauley Thomas G. Griffiths II Laural B. Harper Carolann M. Beare
Walter J. Bagdon Warren W. Nichols

Department of Safety Assessment, Merck Research Laboratories, West Point, Pa., USA

Key Words
DNA vaccine · Vaccination, genetic · Plasmid · Recombination

Abstract
The primary safety concern for DNA vaccines is their potential to integrate into the host cell genome. We describe an integration assay based on purification of high-molecular-weight genomic DNA away from free plasmid using gel electrophoresis, such that the genomic DNA can then be assayed for integrated plasmid using a sensitive PCR method. The assay sensitivity was approximately 1 plasmid copy/μg DNA (representing ~150,000 diploid cells). Using this assay, we carried out integration studies of three different plasmid DNA vaccines, containing either the influenza hemagglutinin, influenza matrix or HIV gag gene. Six weeks after intramuscular injection, free plasmid was detected in treated muscle at levels ranging from approximately 1,000 to 4,000 copies/μg DNA. At 6 months, the plasmid levels ranged between 200 and 800 copies/μg DNA. Gel purification of genomic DNA revealed that essentially all of the detectable plasmid in treated quadriceps was extrachromosomal. If integration had occurred, the frequency was ≤1–8 integrations per 150,000 diploid cells, which would be at least three orders of magnitude below the spontaneous mutation rate. Our results suggest that the risk of mutation due to integration of plasmid DNA vaccines following intramuscular injection is negligible.

Introduction

DNA vaccines represent a promising new approach to vaccination in which the gene for a foreign antigen is expressed within the host's cells. The antigen gene is delivered within a DNA expression vector, which contains transcriptional promoter and polyadenylation signals that permit the expression of the gene in mammalian cells. In most cases, plasmids have been used as the expression vector, since they are chemically simple, they are relatively easy to produce and characterize, and they can be delivered by a variety of routes either as naked DNA or complexed with lipid or gold particles [1]. Plasmids contain bacterial origins of replication so that they can be propagated in laboratory strains of *Escherichia*

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Brian J. Ledwith, PhD
Merck Research Laboratories
West Point, PA 19486 (USA)
Tel. +1 215 652 4319, Fax +1 215 652 7758
E-Mail brian_ledwith@merck.com

coli, but they usually do not contain eucaryotic origins of replication and thus cannot replicate in mammalian cells.

Intramuscular injection is often used to deliver DNA vaccines because it results in relatively efficient uptake and long-term expression of the transgene [1, 2]. Following injection, plasmid DNA is taken up by muscle cells and the gene encoding the foreign antigen is expressed using the cells' transcription and translation machinery. Expression of the antigen in vivo permits the induction of both a humoral and a cell-mediated immune response. Neutralizing antibodies and cross-strain protective cytotoxic T lymphocytes have been induced in animal models using DNA vaccines to a wide variety of viral, bacterial, parasitic and tumor antigens [1, 3].

The primary safety concern for DNA vaccines is their potential to integrate into the host cell genome [4, 5]. Integration is by definition insertional mutagenesis and has the potential to activate oncogenes or inactivate tumor suppressor genes. Plasmids are known to integrate into cellular DNA when transfected into actively dividing cells in vitro. The frequency of plasmid integration in vivo is expected to be markedly lower than in model systems in vitro, since there are greater barriers to cellular uptake including fibrous tissue architecture and extracellular nucleases [6]. When plasmid integration occurs, the integrated plasmid is found at essentially random sites in the genome [7]. Even in gene-targeting experiments optimized for homologous recombination, random integration is about 50–2,000 times more frequent than integration via homologous sequences [8].

Wolff et al. [2] demonstrated that plasmids can persist in mouse muscle up to 19 months after injection, and that the plasmids remain predominantly extrachromosomal. No evidence of integration was found, but the methods used were relatively insensitive. For safety assessment of DNA vaccines to be used in a clinical setting, a more rigorous integration assay was required. Previously, we reported the development of a highly sensitive assay to study plasmid integration in vivo [9]. The method is based on purification of high-molecular-weight (HMW) genomic DNA away from free plasmid using gel electrophoresis, such that the genomic DNA can then be assayed for integrated plasmid using a sensitive polymerase chain reaction (PCR) procedure. Here, we describe the integration assay in detail, validate the effectiveness of the assay using various positive and negative controls, and provide the results of intramuscular integration studies of three different DNA vaccine plasmids.

Methods

Plasmids

Plasmid constructs were made in variations of the V1Jn vector [10, 11], which is about 4.9 kb in size and contains the enhancer, promoter and intron A sequences from the cytomegalovirus E1 gene (CMVintA promoter), the polyadenylation signal from the bovine growth hormone gene (BGH terminator), the kanamycin resistance gene, and a pUC plasmid backbone. In V1Jns, a 13-bp linker containing an *Sfi*I restriction site was inserted into a *Kpn*I site within the BGH terminator (the polyadenylation signal was not affected). In V1Jp, a 34-bp linker containing two *Sfi* sites (separated by a *Pac*I site) was inserted in the same location.

Three DNA vaccines were tested: V1Jp-HA, a 6,614-bp plasmid containing the hemagglutinin (HA) gene of influenza A/Georgia; V1Jp-M1, a 5,674-bp plasmid containing the matrix (M1) gene of influenza A/Beijing, and V1Jns-tPA-gag, a 6,375-bp plasmid containing the HIV gag gene with a 5′-secretory signal from tissue plasminogen activator. Plasmids were purified by high pressure liquid chromatography (Lee and Sagar, PCT International Application No. WO96/36706). Plasmid purity and stability were established through rigorous analytical testing, including assays for percent supercoiled, restriction fragment pattern, 260 nm/280 nm absorbance ratio, sterility, endotoxin, *E. coli* DNA, single-stranded DNA, RNA, protein, and residual reagents. Each plasmid was sequenced in its entirety, demonstrated to express the appropriate gene product in transient transfection assays, and demonstrated to induce immune responses in mice and other animals [11–13].

For in vitro transfection experiments, the selectable plasmid V1Jp-Hygro (5,927 bp in size) was constructed by inserting a 1,037-bp fragment containing the hygromycin resistance (Hygro) gene into the *Bgl*II site of the V1Jp vector. The Hygro gene fragment was obtained from the pDR2 plasmid (Clontech) using PCR amplification. This plasmid was purified by CsCl centrifugation as described previously [11].

Animal Treatment and Tissue Collection

Balb/c mice (Charles River Laboratories), approximately 6–7 weeks of age, were injected in *both* quadriceps with 50 µl of either plasmid or vehicle (saline or phosphate-buffered saline). For the V1Jp-HA and V1Jp-M1 studies, the mice received two doses, 3 weeks apart, of 100 µg per quadriceps and were necropsied at 9 and 26 weeks (6 and 23 weeks after the second dose). For the V1Jns-tPA-gag study, mice received one dose of 160 µg per quadriceps and were necropsied 6 weeks after injection.

At necropsy, mice were anesthetized using isoflurane and bled via the vena cava into EDTA-containing tubes. The blood was stored at –70°. To assess the tissue distribution of the plasmid, described in the accompanying manuscript [14], multiple tissues were removed from each animal. Quadriceps were removed last to avoid contamination of other tissues. Tissues were rinsed in phosphate-buffered saline, placed in cryotubes, frozen in liquid nitrogen, placed in storage boxes, sealed in plastic bags, and stored at –70° until DNA isolation.

General Procedures for Contamination Control

Because the PCR assay can detect a single copy of plasmid and the mice were injected with $>10^{13}$ copies, stringent procedures were implemented to prevent contamination.

To avoid cross-contamination of tissues during necropsy, animals were necropsied on clean bench paper at individual stations with two people per station, one person performing the dissection and the other processing the tissue. After each round of necropsy, all stations were cleaned with 10% Clorox and then supplied with fresh materials. Materials and reagents were labeled and dispensed into individual aliquots prior to necropsy. A separate set of sterile dissecting instruments was used for each animal, and the instruments were rinsed in 10% Clorox and then double-rinsed with water between tissues. Gloves were changed whenever contaminated and always between animals.

Each major part of the DNA analysis (DNA isolation, gel purification, PCR reaction set-up, and PCR product analysis) was carried out in a separate, dedicated laboratory. Whenever possible, samples were processed under laminar flow hoods. Laboratory areas and equipment were decontaminated with 10% Clorox before and after each use. Disposable materials were used when possible. Nondisposable materials were decontaminated with Clorox (and autoclaved if possible). Materials and reagents were dispensed into individual aliquots in a separate clean room. Positive displacement pipettes were used to transfer small volumes of samples. Disposable protective laboratory attire was worn and gloves were changed frequently. A limited number of samples were processed at a time, and great care was used to prevent cross-contamination. To monitor for contamination, negative control samples were processed alongside treated samples during each step of the assay.

DNA Isolation

DNA was isolated using a 'total DNA' preparation method, which recovers free plasmid along with cellular DNA. In initial studies, a 'nuclear DNA' preparation method was also used, in an attempt to remove free plasmid and facilitate the integration assay. However, the nuclear isolation did not reduce the level of free plasmid, either because the method was only a crude NP40 fractionation or because the plasmid was predominantly nuclear in location [15].

To prepare enough DNA for multiple rounds of gel purification, DNA was isolated from a pool of quadriceps taken from 5 cohort mice. Five frozen quadriceps (~ 1 g total) were homogenized in 18 ml of TEN buffer (10 m*M* Tris, pH 7.5, 25 m*M* EDTA, 100 m*M* NaCl). Homogenization was carried out in a dedicated laminar flow hood using a glass vessel and motor-driven Teflon pestle at about 2,000 rpm, until all major tissue clumps were dispersed. A separate sterile vessel/pestle set was used for each sample.

To each homogenate, about 1/50 volume of 10% sodium dodecyl sulfate (SDS) and about 1/50 volume of 10 mg/ml RNase were added. After a 1-hour incubation at 37°, an additional 1/25 volume of 10% SDS and 1/50 volume of 15 mg/ml proteinase K were added, and the samples were incubated at 50° for 3 h with occasional mixing. Afterwards, the samples were chilled on ice for 10 min and then extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and once with an equal volume of chloroform:isoamyl alcohol (24:1), using PhaseLock tubes as recommended by the manufacturer (5-Prime to 3-Prime, Inc.). DNA was precipitated by the addition of 1/10 volume of 3 *M* sodium acetate and 1 vol of isopropanol and then pelleted by centrifugation for 5 min at approximately 3,000 *g*. The pellet was rinsed with 70% ethanol and then resuspended in TE buffer (10 m*M* Tris, pH 7.5, 1 m*M* EDTA) by gently rocking overnight at 37°.

DNA concentration was determined by absorbance at 260 nm. The 260 nm/280 nm absorbance ratio for each sample was confirmed to be ≥ 1.8. Each sample was analyzed by electrophoresis in a 0.5% agarose gel to ensure that the DNA migrated predominantly as a HMW band and exhibited no detectable RNA contamination.

Preparative Agarose-Gel Electrophoresis

To optimize the separation of genomic DNA from various conformations of free plasmid, three different procedures for conventional electrophoresis (in 20 cm × 25 cm gels) were used: the TAE procedure, using 0.5% agarose (FMC SeaKem Gold) gels in Tris-acetate-EDTA (TAE) buffer, electrophoresed at 40 V for 22.5 h; the TBE procedure, using 1% agarose (FMC SeaKem Gold) gels in Tris-borate-EDTA (TBE) buffer, electrophoresed at 50 V for 45 h (V1Jp-HA study) or 70 V for 32 h (V1Jp-M1 and V1Jns-tPA-gag studies), and the *Gibco*TBE procedure, using 1.2% agarose (Gibco/BRL) gels in TBE buffer, electrophoresed at 80 V for 22 h.

Genomic DNA was also purified by pulsed-field gel electrophoresis (PFGE) using the BioRad CHEF (Clamped Homogeneous Electric Field) Mapper System. For the CHEF procedure, the electrophoresis parameters were determined by the instrument using an auto-algorithm for separation of 10–50 kb DNA. Briefly, 1% agarose (FMC SeaKem Gold) gels in 0.5 × TBE buffer were electrophoresed at 14° for 25 h and 14 min, using a forward voltage gradient of 9 V/cm, reverse voltage gradient of 6 V/cm, initial switch time of 0.3 s, final switch time of 0.92 s, and a linear ramp. In some cases, preparative PFGE was carried out using a BioRad FIGE (Field Inversion Gel Electrophoresis) Mapper system. For the FIGE procedure, optimized for resolving 10–37.5 kb DNA, 1% agarose (FMC SeaKem Gold) gels in 0.5 × TBE buffer were electrophoresed for 22.5 h using a forward voltage of 180 V, reverse voltage of 120 V, initial switch time of 0.1 s, and final switch time of 0.6 s.

The first round of gel purification generally used the TAE procedure. Approximately 5 μg of DNA was loaded per lane, using a 12-well × 2-mm thick comb, such that up to 60 μg could be loaded per gel. Only one sample was run in each rig. Negative control DNA was run in a separate rig to monitor for contamination. After electrophoresis, gels were stained with ethidium bromide. The band of HMW genomic DNA across all lanes was excised as a single band, cutting tightly around the predominant band to minimize contamination with plasmid. For multiple rounds of purification, the TAE and TBE (or *Gibco*TBE) procedures were alternated. The excised band of genomic DNA from one round was often directly subjected to the next round without elution from the gel. The gel slice was placed upside-down at the top of a clean gel plate and a new gel was poured around it. For the CHEF or FIGE procedure, about 3 μg of DNA was loaded per well, usually using DNA that had been previously purified by the TAE and TBE procedures.

Gel slices were placed in dialysis bags and electroeluted (1 sample per rig) in TAE buffer, usually at 50 V for 20 h. The eluted DNA was concentrated using Centricon-30 filtration units (Amicon) as directed by the manufacturer. The buffer was exchanged by diluting into TE buffer and then reconcentrating.

In some experiments, DNA was digested with *Sfi*I restriction enzyme to facilitate the removal of concatemers by gel purification. Digestions were carried out for 3 h at 50° using 10 units of *Sfi*I/μg of DNA. Electrophoresis was then carried out as described above. When *Sfi*I was used, a broader gel slice was excised for electroelution, with a bottom edge around 15 kb.

Table 1. PCR primers

Segment	Oligonucleotide primers	Product size, bp
CMV-III	5′-GCC AAG AGT GAC GTA AGT ACC GCC 5′-GTC CGT GTC AGT CTC TGA AGG ACA	278
BGH-II	5′-GAG GAT TGG GAA GAC AAT AGC AGG 5′-GGG AGA GAC CGC TCC AAG TAC TTT	240
HA-I	5′-CCC GGA AAT GAC AAC AGC ACA GCA 5′-GTG CCT GAT GAG GCA ACT AGT GAC	341
HA-III	5′-CAG GGA ATC TAA TTG CTC CTC GGG 5′-TGC TGC TTG TCC TGT GCC CTC AGA	353
M-II	5′-GTG AGC GAG GAC TGC AGC GTA GAC 5′-GAC CTG TGC TGG GAG TCA GCA ATC	274
GAG-I	5′-GCG AGA TCT CCA TTG TGT GGG CCT 5′-TGC ACA ATG GGG TAG TTC TGG GAC	319
TAG-I	5′-CAT GAA CAG ACT GTG AGG ACT GAG 5′-GTA CAG ACC TGT GGC TGA GTT TGC	302

Characterization of Gel-Purified Genomic DNA

The concentration of gel-purified samples was determined by spotting on ethidium bromide/agarose plates and comparing the fluorescence with that of known standards, or by spectrophotometry using the Pharmacia GeneQuant II. The size of genomic DNA samples before and after gel purification was determined by analytical PFGE using a BioRad CHEF Mapper. Approximately 200 ng of each sample was loaded per lane. Analytical PFGE conditions were determined using an autoalgorithm for separation of 5–250 kb DNA. Following electrophoresis, gels were stained in ethidium bromide and photographed on a UV transilluminator.

PCR Analysis

Generally, DNA samples were assayed for at least three segments of the relevant plasmid, including the CMV-III segment of the cytomegalovirus promoter, the BGH-II segment of the BGH terminator, and one or more segments of the antigen gene (the HA-I and HA-III segments of the HA gene, the M-II segment of the matrix gene, or the GAG-I segment of the gag gene). For gel-purified samples where material was limited, only 1 or 2 segments were assayed. To optimize sensitivity, PCR segments were relatively short (<400 bp). The oligonucleotide primers for each segment, and the expected product size, are listed below. The primers used for amplifying a T antigen gene segment (TAG-I) for the SV40-positive control experiment are also shown (table 1).

Prior to PCR, DNA samples were heat-denatured at about 94° for 5–15 min. PCR reactions were carried out in a final volume of 100 µl containing the following reagents: 10 m*M* Tris, pH 8.3, 50 m*M* KCl, 1.0 or 1.5 m*M* $MgCl_2$, 0.01% (w/v) gelatin, 200 µ*M* of each dNTP, 250 ng of each oligonucleotide primer, 2.5 units of Taq DNA polymerase (Perkin-Elmer Cetus), 1 unit of Perfect Match DNA Enhancer (Stratagene), 0–10% glycerol, and 1 µg of template DNA. In a dedicated laminar flow hood that was never exposed to DNA, the mixture without template DNA was added to reaction tubes on ice, overlaid with mineral oil, and capped. The tubes were transferred to a second hood where template DNA was added. Tubes were taken directly from ice to a thermal cycler that was prewarmed to 94°. The following successive PCR thermal cycle conditions were used: 2 min time delay at 94°; 40 step cycles of 1 min denaturation at 94°, 2 min annealing (at 65–68° for the first 5 cycles, 62–65° for the next 5 cycles, and 60° for the remaining 30 cycles), and 2 min extension at 72°; 7 min time delay at 72°, and then a soak at 4°.

Each PCR experiment included a positive control titration curve consisting of 0, 1, 2, 4, 8 and 16 copies of plasmid, each diluted in 1 µg of mouse DNA. The 0 copy standard served as a negative control to detect contamination. In addition, several H_2O (no DNA)-negative control reactions were included in each experiment. When gel-purified DNA samples were being assayed, negative control DNA that was gel-purified alongside the sample was also assayed. All samples and controls were assayed in duplicate. Following PCR, a 10-µl aliquot of each reaction was analyzed by electrophoresis in a 2% agarose gel. PCR products were stained with ethidium bromide and either photographed on a UV transilluminator or scanned using a Molecular Dynamics FluorImager 595SI.

Dilution/PCR Analysis

To estimate the level of plasmid, treated quadriceps DNA samples were diluted into control mouse DNA such that the PCR products obtained would fall within the range of the positive control titration curve. Samples were diluted at various ratios ranging from 1:10 to 1:1,600, with the overall concentration of DNA remaining constant. Duplicate 1-µg aliquots of each dilution were assayed by PCR for three plasmid segments. The plasmid levels in dilutions were estimated by comparison of their PCR product intensities with those of the positive control titration curve. The values were multiplied by the dilution factor, and the results for different dilution/segment assays of a given sample were then averaged. Assays in which the PCR products fell outside the standard curve were not used.

In vitro Transfection Experiment

The V1Jp-Hygro plasmid was transfected into C_2C_{12} mouse myoblast cells using calcium phosphate precipitation. Individual colonies of cells were isolated and propagated in the presence of hygromycin to obtain stable transfectants with integrated V1Jp-Hygro plasmid. Total DNA was isolated from the hygromycin-resistant clones, digested with the *Sfi*I restriction enzyme, and electrophoresed on a

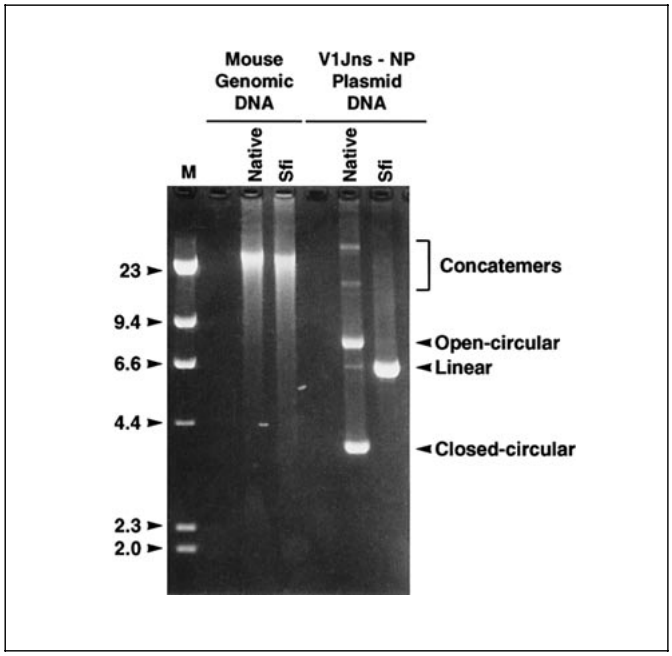


Fig. 1. Purification of genomic DNA from free plasmid using *Sfi*I digestion and gel purification. Mouse genomic DNA and V1Jns-NP plasmid (which contains the influenza nucleoprotein gene and was used in a previous exploratory study [9]) were electrophoresed in a 0.8% agarose gel in TBE buffer. Both DNA samples are shown both uncleaved (native) and cleaved with *Sfi*I restriction enzyme (Sfi). *Hind*III-cleaved λ DNA are shown as size markers (M). The various conformations of uncleaved plasmid are labeled on the right. In the uncleaved samples, concatemers of the plasmid migrate near the position of HMW genomic DNA. After *Sfi*I digestion, all of the plasmid migrates as linear monomers, well separated from the genomic DNA.

0.5% agarose TAE gel as described above. The size of *Sfi*I fragments containing plasmid DNA was analyzed by Southern blotting using ^{32}P-labeled V1Jp-Hygro plasmid as probe.

Results

Use of Gel Purification to Distinguish Free from Integrated Plasmid

HMW genomic DNA was purified away from free plasmid using agarose gel electrophoresis. Genomic DNA, isolated from tissue using standard procedures, consisted predominantly of fragments between 50 and 200 kb in length, which migrated in a conventional agarose gel as an unresolved band (see fig. 1) that could be conveniently excised during gel purification. The genomic DNA was recovered from the gel slice using gentle electroelution and filtration procedures, which minimized shearing of the DNA and avoided contaminants that might inhibit the PCR assay.

Yields of HMW genomic DNA after gel purification (electroelution) were usually between 20 and 50% of starting DNA. Nonspecific losses of DNA during gel purification do not affect the sensitivity of detecting integrated plasmid, since the assay measures copies of plasmid per microgram of genomic DNA. Using cell line DNA labeled with ^{3}H-thymidine in culture, we estimated that ≥60% of the DNA was in the HMW band. The presence of DNA outside the HMW band was due to random shearing or due to trailing and trapping in the gel during electrophoresis. Inefficiencies in the recovery of DNA from the excised gel slice resulted in additional losses. Importantly, all of these DNA losses were nonspecific because they were due to random processes, and thus the HMW genomic DNA recovered after gel purification could be considered representative of the whole (except when restriction enzyme digestion was used, as discussed below).

Since the PCR assay itself does not distinguish free from integrated plasmid, efficient separation of free plasmid from the genomic DNA during gel purification was required. Free plasmid DNA exists in a variety of conformations, including closed-circular supercoiled, open-circular, linear, and various large concatemers. Two or more large concatemers are usually visible by ethidium bromide staining (fig. 1), and additional concatemers can be observed by Southern blotting (not shown). Some free plasmid concatemers could comigrate with HMW genomic DNA during gel purification and produce a false-positive result in the PCR assay. We used two separate approaches for removing these concatemers: restriction enzyme digestion [9] and modulation of electrophoresis conditions.

Use of Restriction Enzyme Digestion

Restriction enzyme digestion was used to cleave free plasmid concatemers into linear monomers [9]. An *Sfi*I restriction site, with a rare 8-bp recognition sequence, was inserted into the plasmid vectors. In a random DNA sequence, the *Sfi*I site would be found on average once in every 4^8 or 65,536 bp, and thus genomic DNA is only minimally affected by *Sfi*I digestion. As shown in figure 1, *Sfi*I digestion cleaves all conformations of the plasmid, including the large concatemers, into linear monomers (about 6 kb) that can be easily separated from genomic DNA. While both free and integrated plasmid would be cleaved by *Sfi*I, integrated plasmid fragments should remain attached to genomic DNA.

Cleavage of genomic DNA with *Sfi*I could result in a partial loss in sensitivity of detecting integrated plasmid, because regions of genomic DNA with integrated plasmid would have an extra *Sfi*I site (donated by the plasmid) relative to genomic DNA in general. Making the conservative assumption that the plasmid donates the *Sfi*I site but no additional size, then fragments containing integrated plasmid would be about half the size of bulk genomic DNA fragments, resulting in a selective loss of integrated plasmid during gel purification. This loss would depend on the size of the genomic DNA fragments and the bottom edge of the excised gel fraction. After serial gel purification and *Sfi*I digestion, the average size of genomic DNA was found by analytical PFGE to be about 30 kb. For *Sfi*I-digested DNA, we usually excised a broad gel slice with a bottom edge around 15 kb. Using these values, it was calculated that approximately 50% of the integrations may be lost, corresponding to a 2-fold reduction in assay sensitivity.

To empirically test the effect of *Sfi*I digestion on integrated DNA, a plasmid containing the hygromycin resistance (V1Jp-Hygro) was transfected into C_2C_{12} mouse myoblasts. Clones containing integrated plasmid were selected by propagation in the presence of hygromycin and analyzed by *Sfi*I digestion and Southern analysis. In such in vitro systems, plasmids often integrate as multiple copies in a single site, and thus a variety of smaller bands representing plasmid-plasmid *Sfi*I fragments were expected in addition to some larger bands representing plasmid-genomic fragments. Representative results are shown in figure 2. Most clones had at least one plasmid-containing fragment that was greater than 15 kb, representing a plasmid-genomic fragment that would be recovered during gel purification. These results support the idea that, while *Sfi*I digestion may partially decrease the sensitivity of detecting integration, a large fraction of the integrations would still be detected.

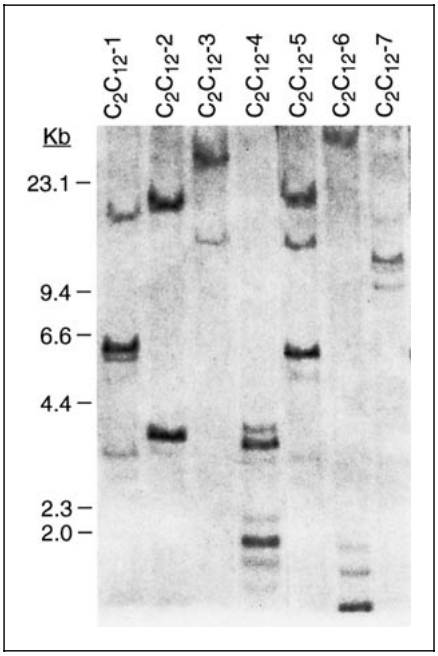


Fig. 2. Southern analysis of integrated plasmid after *Sfi*I digestion. V1Jp-Hygro plasmid was transfected into C_2C_{12} cells, and clones containing integrated plasmid were selected by propagation in medium containing hygromycin. DNA from each clone was cleaved with *Sfi*I and analyzed by Southern analysis using a ^{32}P-labeled V1Jp-Hygro plasmid as probe. The *Sfi*I fragments containing integrated plasmid were visualized by autoradiography. Representative clones are shown. Fragments containing integrated plasmid that are <15 kb would be lost, those >15 kb would be recovered during gel purification.

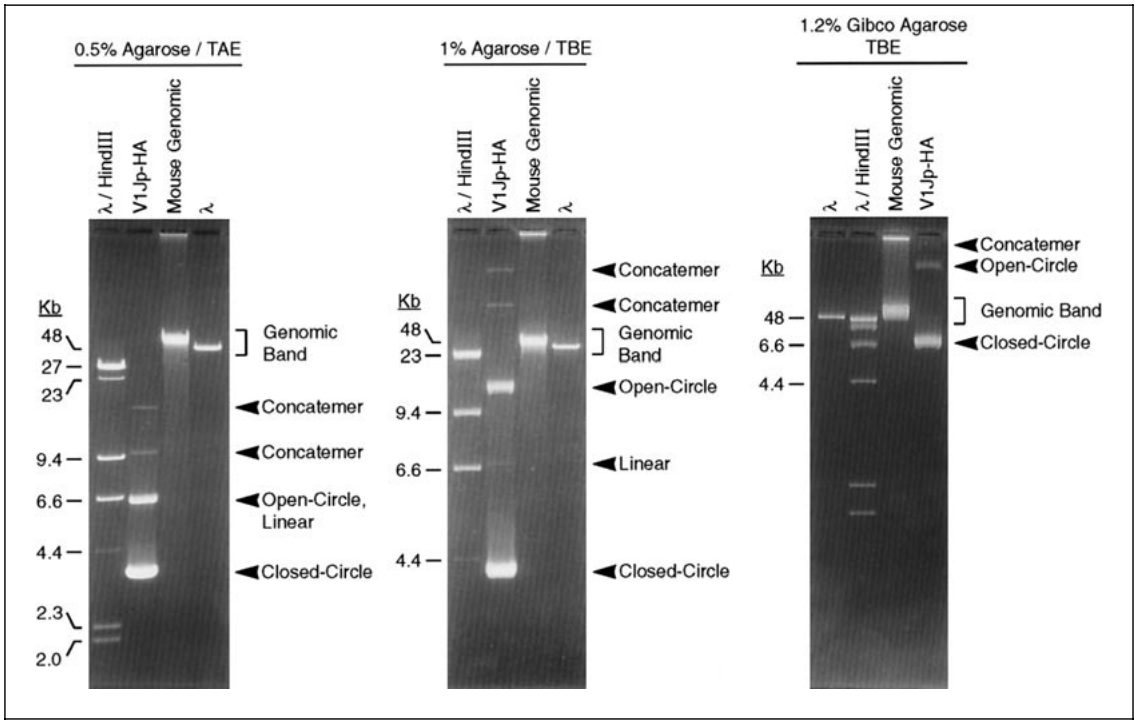
Differential Electrophoresis Conditions for Separating Free Plasmid from Genomic DNA

We noticed that variations in the electrophoresis conditions affected the migration of *circular* plasmids relative to *linear* genomic DNA. Most of the concatemers present in plasmid preparations appeared to be circular species. By using a variety of gel electrophoresis procedures, all of the major conformations of the plasmid could be separated from the genomic DNA without using restriction enzyme digestion.

The migration of circular plasmids relative to linear genomic DNA was affected by (1) the concentration of agarose – circular forms migrate as a smaller apparent size in lower percentage agarose; (2) the type of agarose – circular forms migrate as a smaller apparent size in FMC SeaKem Gold versus Gibco agarose; (3) the electrophoresis buffer – circular forms migrate as a smaller apparent size in TAE than in TBE buffer, and (4) the electric field strength – circular forms migrate as a smaller apparent size with lower voltage/centimeter.

By varying these factors, three procedures for conventional agarose gel electrophoresis were developed (fig. 3). In the TAE procedure, the major concatemers migrated *ahead* of the genomic band. In the TBE procedure, the major concatemers migrated *behind* the genomic band. In the *Gibco*TBE procedure, the major concatemers, as well as open-circular monomers, migrated *behind* the genomic band. The precise electrophoresis conditions in each procedure were optimized for each different plasmid.

In addition to the conventional electrophoresis procedures, we also used a preparative pulsed-field gel electro-



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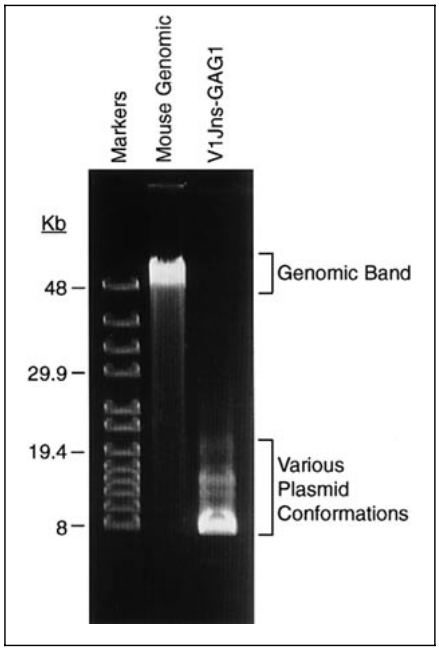

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Fig. 3. Separation of plasmid from genomic DNA using differential electrophoresis conditions. The TAE, TBE and *Gibco*TBE procedures for agarose gel electrophoresis were carried out as described in Methods. Each panel shows mouse genomic DNA (1 μg), V1Jp-HA plasmid (1 μg), and size markers (intact λ DNA and *Hin*dIII-cleaved λ DNA) as indicated. In the TAE procedure (0.5% SeaKem agarose gels in TAE buffer at 40 V), the circular forms of V1Jp-HA plasmid, including the two major concatemers, migrate well ahead of the band of HMW genomic DNA (note: the open-circular form comigrates with the linear form). In the TBE procedure (1% SeaKem agarose gels in TBE buffer at 50 V), the circular forms of V1Jp-HA plasmid migrate as a higher apparent size, such that the two major concatemers migrate well behind the band of HMW genomic DNA. In the *Gibco*TBE procedure (1.2% Gibco agarose gels in TBE buffer at 80 V), even open-circular monomers migrate behind the band of HMW genomic DNA.

Fig. 4. Preparative CHEF PFGE. The CHEF procedure for preparative pulsed-field electrophoresis was carried out as described in Methods, loading 2 μg of genomic DNA and 1 μg of V1Jp-HA plasmid. The genomic DNA had previously been subjected to 4 rounds of gel purification (using the procedures described in fig. 5). Size markers (8–48 kb) are shown. All of the major plasmid forms migrate well ahead of the band of HMW genomic DNA.

phoresis procedure, referred to as CHEF. In the CHEF procedure, all conformations of the plasmid migrated well ahead of the genomic band (fig. 4). In addition, there was better separation of genomic DNA from linear fragments between 20 and 50 kb, and thus this procedure would be more effective than conventional gels at removing linear plasmid concatemers if they were present.

For samples with high plasmid levels, multiple rounds of gel purification were necessary. Usually, the TAE and TBE (or *Gibco*TBE) procedures were alternated, since

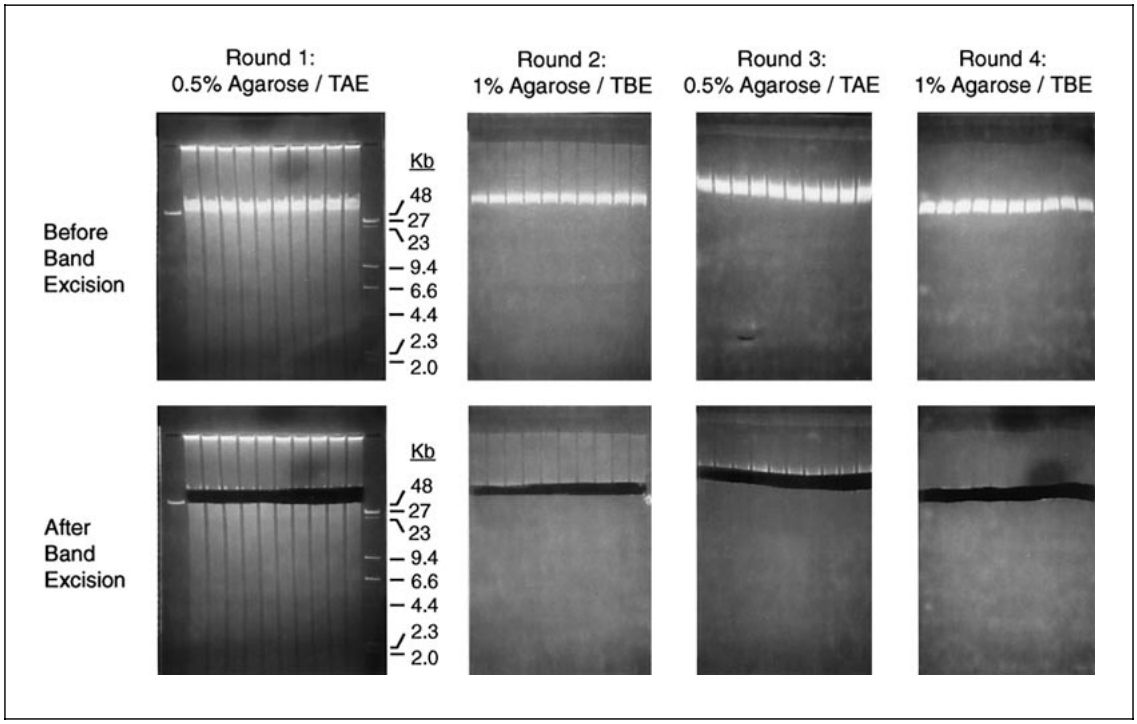


Fig. 5. Multiple-round gel purification. Control mouse genomic DNA was subjected to 4 consecutive rounds of gel purification without electroelution between rounds. For the first round, 5 μg of genomic DNA was loaded into each of 10 lanes of a 0.5% agarose TAE gel (see Methods). After electrophoresis, the band of HMW genomic DNA was excised as a single band. Since the HMW band is representative of the whole, a narrow gel slice was excised around the band to avoid trailing plasmid. The gel slice was placed upside-down at the top of a clean gel plate, and a 1% agarose TBE gel was poured around it for round 2. The process was repeated for a TAE gel in round 3 and a TBE gel in round 4. After the final round, HMW genomic DNA in the excised band was electroeluted. The gels are shown before and after band excision. Size markers were run on the first gel (lane 1: λ DNA, lane 12: λ/*Hin*dIII DNA).

concatemers which comigrate with genomic DNA under one procedure would be removed using another procedure. To minimize DNA losses and shearing that occurs during the electroelution and re-concentration of DNA, the excised band of HMW genomic DNA from one round was often directly subjected to the next round without elution from the gel (illustrated in fig. 5).

Detection of Plasmid by PCR

PCR was used to quantitate free plasmid in tissue DNA samples and to assay for integrated plasmid in gel-purified genomic DNA. Each PCR assay contained several negative controls as well as a reconstruction positive control titration curve. The level of plasmid in test samples was estimated by comparison of their PCR products with those of the standard curve. The sensitivity of detecting plasmid was routinely 1 copy/μg DNA (in at least one of two duplicate reactions). Because of the sensitivity of the PCR reaction and the fact that mice are injected with large amounts of plasmid ($>10^{13}$ copies), stringent measures were implemented to control contamination during all parts of the integration assay (see Methods). Although artifacts were relatively rare, assays were repeated if the sensitivity was not 1–2 copies/μg, if nonspecific 'background' bands or primer dimers appeared, or if contamination was evident in negative controls. Whenever a repeat assay was performed, all samples in the assay were repeated, not just the questionable sample.

It was important to demonstrate that tissue DNA preparations did not contain inhibitors of the PCR reaction. First, each DNA preparation was shown to be of high purity and integrity by spectrophotometry and analytical gel electrophoresis (see Methods). In addition, control DNA was isolated from a variety of tissues (including quadriceps) and then used to prepare a positive control titration curve containing 1–16 copies of V1Jp-HA plasmid. The titration curve was also prepared in gel-purified DNA. These titration curves were then assayed by PCR for the

CMV-III and HA-III segments of the plasmid. In all cases, the sensitivity of detecting plasmid was 1–2 copies/μg DNA, similar to that observed with the routine positive control curve prepared in DNA isolated from C_2C_{12} mouse myoblast cells. These results confirm the absence of significant PCR inhibitors in tissue DNA preparations or in gel-purified DNA.

Gel Purification Is Effective at Removing Free Plasmid when Spiked in vitro

To demonstrate that the gel purification method is effective at removing free plasmid from genomic DNA, we performed a trial experiment in which control mouse DNA was spiked in vitro with 5,000 copies of V1Jp-HA plasmid/μg DNA. The genomic DNA was then subjected to 4 rounds of gel purification (using the same procedures shown in fig. 5) and assayed by PCR in duplicate for 4 different segments of the plasmid: BGH-II, CMV-III, HA-I and HA-III. All assays were negative to a sensitivity of 1 copy/μg DNA, except for one of the two duplicates in the HA-III assay which was faintly positive (<1 copy). These results demonstrated that the gel purification method was highly effective at removing free plasmid from genomic DNA when the plasmid was spiked in vitro.

Positive Control Using Integrated SV40 DNA

To demonstrate that integrated DNA could be detected by the assay, a positive control experiment was performed using COS-1 cells. These well-characterized cells, developed by transfection of CV-1 monkey kidney cells with origin-defective SV40, are known to contain a single integrated copy of the SV40 early region [16]. Unlike vaccine plasmids, the SV40 DNA in COS-1 cells does not contain an *Sfi*I site (the site at position 5241 was destroyed while making the origin-defective mutant [17]). Thus, the COS-1 control does not assess the selective loss of integrated DNA due to *Sfi*I digestion, but it does control for all other aspects of the integration assay.

DNA was isolated from COS-1 and CV-1 cells using our standard procedures. The COS-1 DNA was diluted into CV-1 DNA at various ratios and then assayed by PCR for a 302-bp segment (TAG-I) of the T antigen gene, using PCR conditions similar to those used for DNA vaccine plasmids. In several replicate experiments, the TAG-I segment was detected in dilutions down to 1 copy/μg DNA.

Two gel purification experiments were carried out. In the first experiment, COS-1 dilutions containing 0, 1, 2 and 10 copies of SV40 DNA/μg DNA were subjected to *Sfi*I digestion followed by 1 round of gel purification in a TAE gel. The HMW genomic band for each dilution was electroeluted and assayed by PCR for the TAG-I segment. Both before and after gel purification, the 0 copy samples were negative and the 1-, 2-, and 10-copy samples were positive (not shown).

In the second experiment, multiple rounds of gel purification were used to mimic our most rigorously treated sample from a DNA vaccine study. COS-1 DNA was diluted into CV-1 DNA such that there were 4 copies of SV40/μg DNA. We then carried out 9 rounds of gel purification, with electroelution after rounds 4, 6, 8 and 9, and digestion with *Sfi*I prior to the 9th round. At each stage of purification (after electroelution), the sample was assayed by PCR for the TAG-I segment. As shown in figure 6, there was no significant difference in the sensitivity of detecting integrated SV40 DNA between the pre-gel sample and the 4 ×, 6 ×, 8 × or 9 × gel-purified samples.

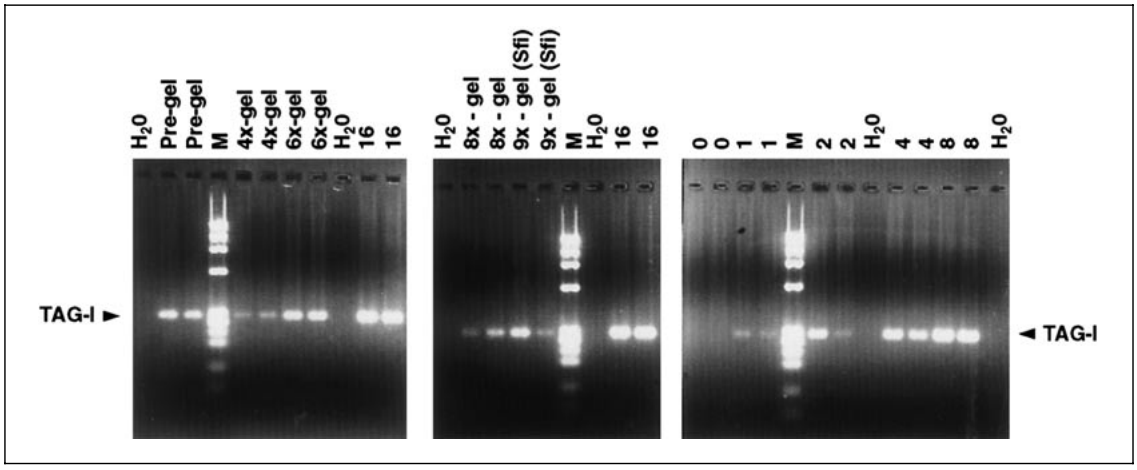
Each gel-purified DNA sample is examined by analytical PFGE to confirm that the DNA remains essentially intact. As an example, figure 7 shows the PFGE analysis of the sample from the SV40-positive control experiment, demonstrating that the DNA remained at HMW throughout the 9 rounds of gel purification.

These experiments demonstrate that the assay can detect integrated DNA with a sensitivity of 1 copy/μg DNA, and that the DNA isolation and gel purification procedures do not significantly reduce the assay sensitivity.

Plasmid Integration Studies in Mice

The potential for integration into host DNA after intramuscular injection in mice was tested for three different DNA vaccine plasmids: V1Jp-HA, containing the influenza HA gene; V1Jp-M1, containing the influenza matrix gene, and V1Jns-tPA-gag, containing the HIV gag gene. All studies used naked plasmid DNA delivered by intramuscular injection. For V1Jp-HA and V1J-M1, mice were dosed twice, 3 weeks apart, with 100 μg per quadriceps and necropsied at 9 weeks (6 weeks after the second dose) and at 26 weeks. For V1Jns-tPA-gag, mice were dosed once with 160 μg per quadriceps and necropsied at 6 weeks.

To have enough material for gel purification, DNA was isolated from a pool of quadriceps taken from 5 cohort mice for each treatment group/sex/time point. All DNA preparations were shown to have A260/A280 ratios of ≥1.8, be of high molecular weight, and be devoid of detectable RNA, and thus they were of excellent quality for PCR. The quadriceps DNA samples were assayed by PCR for 3–4 plasmid segments. The sensitivity of the



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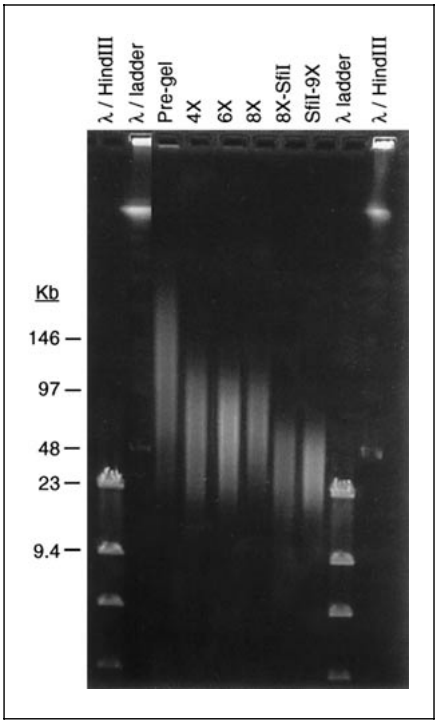

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Fig. 6. PCR analysis of integrated SV40 DNA before and after gel purification. COS-1 cell DNA was diluted into CV-1 cell DNA such that there were about 4 copies of SV40 DNA/μg genomic DNA. The dilutions were assayed in duplicate before (Pre-gel) and after various rounds (4 ×, 6 ×, 8 × or 9 ×) of gel purification. *Sfi*I digestion (Sfi) was performed on the sample prior to the 9th round of gel purification. The SV40 T antigen gene segment, TAG-I, was assayed using PCR assay conditions similar to those used for the vaccine plasmid segments. A titration curve of COS-1 DNA in CV-1 DNA consisting of 0, 1, 2, 4, 8 and 16 copies of SV40/μg DNA, and H_2O negative controls are also shown.

Fig. 7. Analytical PFGE of gel-purified DNA. Mouse genomic DNA from the positive control experiment described in figure 6 was analyzed by analytical PFGE using a BioRad CHEF Mapper and an auto-algorithm for separation of 5–250 kb DNA. Aliquots of the DNA prior to gel purification (Pre-gel) and after various rounds (4 ×, 6 ×, 8 × or 9 ×) of gel purification were analyzed. *Sfi*I digestion was performed on the sample prior to the 9th round of gel purification. The 8 ×-*Sfi*I sample was analyzed after *Sfi*I digestion but prior to the 9th round of gel purification; *Sfi*I-9 × represents the sample after the 9th round. Each lane contains 200 ng of DNA. A 50-kb ladder and λ/*Hin*dIII size markers are shown.

PCR reaction was routinely 1 copy of plasmid/μg DNA. To estimate the level of plasmid in treated quadriceps DNA, the samples were diluted into control mouse DNA such that their PCR product intensities fell within those of the standard curve (1–16 copies/μg DNA). An example of the dilution/PCR analysis is shown in figure 8. The results for each study are shown in table 2. All quadriceps DNA samples from vehicle control mice were negative for plasmid. In quadriceps from treated mice, the level of plasmid at 6 or 9 weeks ranged from about 1,000 to about 4,000 copies/μg DNA. Significant levels of plasmid were still detected at 26 weeks in the V1Jp-HA and V1Jp-M1 studies, ranging from about 200 to about 800 copies/μg DNA.

To determine if there was integrated plasmid, HMW genomic DNA was gel-purified and assayed by PCR. Treated quadriceps DNA samples from both the 6- or 9-week and 26-week time points were analyzed. Representative results for each study are shown in table 3. An example of the PCR results is shown in figure 9. The integrity of gel-purified samples was determined by analytical PFGE (see fig. 7 for example), and in all cases the

Table 2. Plasmid levels in quadriceps DNA following intramuscular injection of mice

DNA vaccine study	Time point weeks	Treatment group	Quadriceps DNA sample name	Approximate plasmid level copies/μg DNA
V1Jp-HA	9	Vehicle control – female	HA-CQ9a	negative
	9	Vehicle control – male	HA-CQ9b	negative
	9	2 mg/ml V1Jp-HA – female	HA-TQ9a[a]	1,600 ± 500
	9	2 mg/ml V1Jp-HA – male	HA-TQ9b[a]	1,800 ± 500
V1Jp-HA	26	Vehicle control – female	HA-CQ26a	negative
	26	Vehicle control – male	HA-CQ26b	negative
	26	2 mg/ml V1Jp-HA – female	HA-TQ26a	280 ± 150
	26	2 mg/ml V1Jp-HA – male	HA-TQ26b	220 ± 120
V1Jp-M1	9	Vehicle control – female	M1-CQ9a	negative
	9	Vehicle control – male	M1-CQ9b	negative
	9	2 mg/ml V1Jp-M1 – female	M1-TQ9a	1,900 ± 1,000
	9	2 mg/ml V1Jp-M1 – male	M1-TQ9b	2,300 ± 1,100
V1Jp-M1	26	Vehicle control – female	M1-CQ26a	negative
	26	Vehicle control – male	M1-CQ26b	negative
	26	2 mg/ml V1Jp-M1 – female	M1-TQ26a	800 ± 500
	26	2 mg/ml V1Jp-M1 – male	M1-TQ26b	700 ± 400
V1Jns-tPA-gag	6	Vehicle control – female	GAG-CQ6a	negative
	6	Vehicle control – male	GAG-CQ6b	negative
	6	3.2 mg/ml V1Jns-tPA-gag – female	GAG-TQ6a	1,400 ± 600
	6	3.2 mg/ml V1Jns-tPA-gag – male	GAG-TQ6b	3,700 ± 1,200

For the V1Jp-HA and V1Jp-M1 studies, mice were dosed twice, on days 1 and 22, and necropsied at 9 weeks (6 weeks after second injection) and at 26 weeks. For the V1Jns-tPA-gag study, mice were dosed once and necropsied at 6 weeks. For each dose, a 50-μl volume was injected into each quadriceps (plasmid concentrations are indicated). Total DNA was isolated from a pool of quadriceps taken from 5 cohort mice for each treatment group/sex/time point. Each DNA sample was assayed by PCR for 3 or 4 segments of the plasmid, including the CMV-III and BGH-II (all studies) as well as either HA-I or HA-III (for V1Jp-HA), M-II (for V1Jp-M1), or GAG-I (for V1Jns-tPA-gag). The PCR assay was sensitive to 1 copy/μg DNA. Treated samples were diluted into control DNA at various ratios from 1:10 to 1:1,600, and each dilution was assayed in duplicate for 3 plasmid segments. Results for each sample were averaged to give the approximate plasmid level.

[a] A nuclear DNA prep was used for the indicated sample, but the amount of plasmid was not less than detected in total DNA prepared from an aliquot of the same homogenate.

DNA samples maintained a high molecular weight (average size of 30–50 kb).

As shown in table 3, gel purification of HMW genomic DNA was able to remove virtually all of the plasmid. For example, sample HA-T9a, with 1,600 copies/μg prior to gel purification, was reduced to ≤1 copy/μg. Further purification through 2 more rounds of gel electrophoresis (HA-TQ9a-6X) did not reduce the plasmid level further. A 7th round of gel purification, preceded by *Sfi*I digestion, only marginally reduced the plasmid level. Similar results were obtained for each of the other treated quadriceps samples tested. In every case, the plasmid level was reduced by gel purification to ≤1–8 copies/μg, and this level of purity was obtained *without* using *Sfi*I digestion, generally in 4–6 rounds of gel purification. Once this level of purity was achieved, additional rounds of purification with or without *Sfi*I digestion resulted in little or no further reduction. These results indicate that virtually all of the plasmid persisting in quadriceps at 6–26 weeks after injection was extrachromosomal.

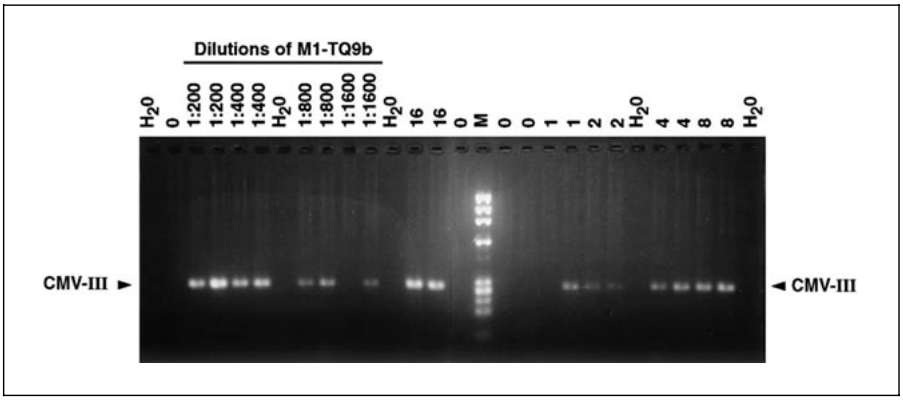

Fig. 8. Quantitation of free plasmid by dilution/PCR analysis. In this example, treated quadriceps DNA sample M1-T9b was diluted into control mouse DNA at ratios of 1:200, 1:400, 1:800 and 1:1,600. The PCR results for the CMV-III segment are shown. The PCR experiment contained several negative controls (H_2O) and a positive control titration curve containing 0, 1, 2, 4, 8 or 16 copies of V1Jp-M1 plasmid in 1 μg of mouse DNA. All samples and controls were assayed in duplicate. PCR products were analyzed by agarose-gel electrophoresis and ethidium bromide staining. The 16-copy reaction products were run on the same gel as the test samples to serve as a marker for the expected product size. ΦX174/*Hae*III markers (M) were run on each gel. The amount of plasmid in each dilution was estimated by comparing its PCR product intensity with those of the positive control titration curve. The estimated plasmid level was multiplied by the dilution factor, and the various segment/dilution assays were then averaged.

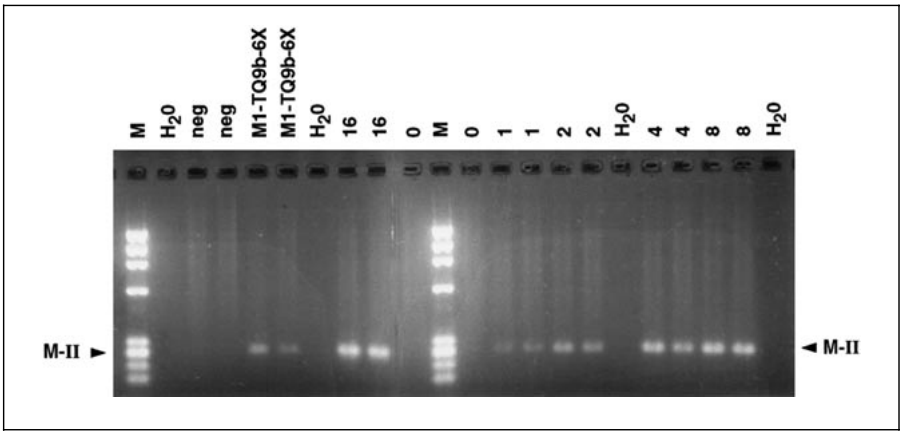

Fig. 9. PCR analysis of gel-purified quadriceps DNA. In this example, treated quadriceps sample M1-T9b-6 ×, purified through 6 rounds of gel purification, was assayed for the M-II segment and was found to contain about 1–4 plasmid copies/μg of DNA. Negative control DNA (neg) gel purified at the same time was also assayed. The PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. The PCR experiment contained several negative controls (H_2O) and a positive control titration curve containing 0, 1, 2, 4, 8 or 16 copies of V1Jp-M1 plasmid in 1 μg of DNA. All samples and controls were assayed in duplicate. The 16-copy reaction products were run on the same gel as the test samples to serve as a marker for the expected product size. ΦX174/*Hae*III markers (M) were run on each gel.

Discussion

In the present report, we describe an assay for investigating the potential integration of plasmid DNA vaccines into host genomic DNA. The assay involves using gel purification to separate free plasmid from HMW genomic DNA, and then assaying the gel-purified genomic DNA for integrated plasmid using a highly sensitive PCR method. The integration assay was validated using a variety of positive and negative controls. The gel purification method was shown to be efficacious at removing free plasmid spiked into mouse genomic DNA in vitro. The PCR assay

Table 3. Gel purification of treated quadriceps

Treatment group	Sample name	Summary of gel purification steps	Segment assayed	PCR results (duplicates)	Approximate plasmid level copies/μg DNA
V1Jp-HA, 9 weeks, female	HA-TQ9a-4 ×	TAE, TBE, TAE, TBE, EE	CMV-III	Pos, Neg	~ 1
			CMV-III	Pos, Neg	
	HA-TQ9a-6 ×	4 × + TAE, TBE, EE	CMV-III[a]	Pos, Neg	~ 1
	HA-TQ9a-7 ×	6 × + *Sfi*I digestion, TAE, EE	CMV-III	Pos, Neg	≤ 1
			HA-III	Neg, Neg	
V1Jp-HA, 9 weeks, female (repeat)	HA-TQ9a-8 × R	TAE, TBE, TAE, TBE, TAE, TBE, EE	CMV-III	Pos, Neg	≤ 1
			HA-III	Neg, Neg	
V1Jp-HA, 9 weeks, male	HA-TQ9b-4 ×	TAE, TBE, TAE, TBE, EE	CMV-III	Pos, Pos	8–16
	HA-TQ9b-6 ×	4 × + TAE, TBE, EE	CMV-III	Pos, Pos	1–8
			CMV-III	Pos, Pos	
	HA-TQ9b-8 ×	6 × + TAE, TBE, EE	CMV-III	Pos, Neg	~ 1
	HA-TQ9b-9 ×	8 × + *Sfi*I digestion, TAE, EE	CMV-III	Neg, Neg	negative
			HA-III	Neg, Neg	
V1Jp-HA, 26 weeks, female	HA-TQ26a-4 ×	TAE, TBE, TAE, TBE, EE	CMV-III	Pos, Pos	4–8
	HA-TQ26a-6 ×	4 × + FIGE, TBE, EE	CMV-III	Pos, Neg	~ 1
			BGH-II	Neg, Neg	
			HA-III	Pos, Pos	
V1Jp-HA, 26 weeks, male	HA-TQ26b-4 ×	TAE, TBE, TAE, TBE, EE	CMV-III	Pos, Neg	~ 1
	HA-TQ26b-5 ×	4 × + FIGE, EE	CMV-III	Pos, Neg	~ 1
			BGH-II	Pos, Neg	
			HA-III	Pos, Neg	
V1Jp-M1, 9 weeks, male	M1-TQ9b-2 ×	TAE, TBE, EE	M-II	Pos, Pos	8–16
	M1-TQ9b-4 ×	2 × + FIGE, TBE, EE	M-II	Pos, Pos	8–16
			CMV-II	Pos, Pos	8–16
	M1-TQ9b-6 ×	4 × + CHEF, TBE, EE	M-II	Pos, Pos	1–4
			CMV-III	Pos, Pos	4–8
V1Jp-M1, 9 weeks, male (repeat)	M1-TQ9b-4 × R	TAE, TBE, TAE, TBE, EE	CMV-III	Pos, Pos	16
	M1-TQ9b-6 × R	4 × + CHEF, TBE, EE	CMV-III	Pos, Pos	4–16
	M1-TQ9b-8 × R	6 × + CHEF, TBE, EE	CMV-III[b]	Pos, Pos	2–4
V1Jp-M1, 9 weeks, male (second repeat)	M1-TQ9b-2 × R2	TAE, *Gibco*TBE, EE	CMV-III	Pos, Neg	1–8
	M1-TQ9b-3 × R2	2 × + CHEF, EE	CMV-III	Pos, Pos	1–8
	M1-TQ9b-4 × R2	3 × + *Gibco*TBE, EE	CMV-III	Pos, Pos	1–8
			M-II	Pos, Pos	1–8
			BGH-II	Pos, Pos	1–8
V1Jp-M1, 26 weeks, male	M1-TQ26b-4 ×	TAE, TBE, TAE, *Gibco*TBE, EE	CMV-III	Pos, Pos	1–4
	M1-TQ26b-5 ×	4 × + CHEF, EE	CMV-III	Pos, Neg	1–2
V1Jns-tPA-gag, 6 weeks, male	GAG-TQ6b-4 ×	TAE, TBE, TAE, TBE, EE	CMV-III	Pos, Neg	4–8
	GAG-TQ6b-5 ×	4 × + *Gibco*TBE, EE	CMV-III	Pos, Neg	1–4
			GAG-I	Pos, Neg	~ 1
	GAG-TQ6b-6 ×	5 × + CHEF, EE	CMV-III	Pos, Pos	2–8
			GAG-I	Pos, Neg	<1
			BGH-II	Pos, Neg	1–2

Treated quadriceps DNA samples were subjected to gel purification to remove free plasmid. Sample names are described in table 2. Suffixes denote the stage of gel purification (e.g., 4 × = 4 rounds of gels; an 'R' indicates a repeat experiment). The series of gel purifications used for each sample is listed. When a sample was further purified and reassayed, the gel purification steps listed begin with the stage previously assayed (e.g., '4 × + ...'). Gel purification steps are abbreviated as TAE, TBE, *Gibco*TBE, CHEF and FIGE as described in Methods. EE refers to when a sample was eluted from the gel by electroelution and represents a stopping point where PCR analysis was carried out. *Sfi*I digestion is indicated when used. Duplicate 1-μg aliquots of the gel-purified DNA were assayed by PCR for the plasmid segments indicated. The PCR assay was sensitive to 1 copy/μg DNA except where indicated. Data for repeat assays of a given segment are shown.

[a] PCR assay was repeated 3 additional times with similar results.

[b] Sensitivity was 2 copies/μg.

sensitivity, using reconstruction positive controls, was consistently about 1 copy of plasmid/μg DNA, equivalent to 1 copy/150,000 diploid genomes. The same sensitivity was obtained when plasmid was spiked into quadriceps samples or into gel-purified DNA, or when an integrated positive control was used (integrated SV40 DNA in COS-1 cells). In addition, when the SV40-integrated positive control was subjected to up to 9 rounds of gel purification, there was no loss in the sensitivity of detecting the integrated DNA. Collectively, these results show that the DNA isolation, gel purification and PCR assay procedures used for the integration assay permit sensitive detection of integrated DNA.

We previously suggested that digestion of DNA with a rare-cutting restriction enzyme such as *Sfi*I can facilitate the removal of plasmid concatemers [9]. However, restriction enzyme digestion may reduce the sensitivity of detecting integrated plasmid by approximately 2-fold, due to the selective loss of integrated plasmid relative to bulk genomic DNA during gel purification. Here, we demonstrated that plasmid concatemers could be removed without restriction enzyme digestion by using a variety of electrophoresis procedures that differentially affect the migration of circular plasmids relative to linear genomic DNA. The migration of circular DNA relative to linear DNA was affected by the agarose concentration, agarose type, electrophoresis buffer, and voltage. In addition, to help remove any linear concatemers that may be present, we used a preparative PFGE method to provide better resolution of 10–50 kb DNA.

The integration assay described here has three major advantages. First, the assay is quantitative, since the approximate plasmid level can be determined before and after gel purification. Second, the PCR assay uses two specific primers for a short plasmid segment, and thus obtains maximum sensitivity. Third, the assay does not involve cloning or ligation steps which could artificially join plasmid to genomic DNA and thereby create false positives.

Intramuscular studies in mice were carried out for three different DNA vaccine plasmids. We found that the plasmid persists in muscle tissue at least 6 months after injection, consistent with the observations of Wolff et al. [2]. Here, using our highly sensitive assay for integration, we showed that virtually all of the plasmid persisting in the muscle is extrachromosomal. Gel purification reduced the level of plasmid in the genomic down to $\leq$1–8 copies/μg DNA, and $\leq$1 copy/μg DNA in some cases.

The residual plasmid that remains with HMW genomic DNA following gel purification could represent integrated plasmid. If the levels were higher, it would be important to confirm integration by amplifying and sequencing the genomic-plasmid DNA junctions (the levels in this study were too low to be analyzed in this way). However, it is also possible that the residual plasmid is extrachromosomal. Purification procedures almost never yield 100% pure material, and 1 copy ($<1 \times 10^{-17}$ g) of plasmid/μg genomic DNA represents an impurity level of <100 billionth by weight. The residual free plasmid could be trapped in the genomic DNA, or it could be large linear concatemers of similar size to genomic DNA such that it comigrates with genomic DNA during electrophoresis.

Even if the residual plasmid in the gel-purified genomic DNA did represent integrated plasmid, it was previously calculated that 1 copy of integrated plasmid/μg of genomic DNA (representing 150,000 diploid cells) would be at least three orders of magnitude below the frequency of spontaneous gene-inactivating mutations [9]. Briefly, 1 integration/150,000 cells would equal 6.7×10^{-6} integrations per cell. Dividing the latter value by 50,000 genes/cell gives a frequency of 1.3×10^{-10} integrations per gene. Increasing this value 10-fold as a worst-case estimate gives a gene mutation frequency of 1.3×10^{-9}, about 1,000 times lower than the spontaneous rate of gene-inactivating mutations, estimated to be approximately 2×10^{-6} mutations per gene [18]. This is a worst-case comparison both quantitatively (10-fold exaggeration) and qualitatively (since many integration events could be innocuous).

We obtained similar results for three different DNA vaccine plasmids delivered by intramuscular injection. However, it is important to individually test each different plasmid, formulation, delivery method, and route of administration, since these factors could potentially affect integration frequency. These factors are examined in more detail in the accompanying manuscript by Manam et al. [14], which also describes the tissue distribution of the plasmid DNA following intramuscular injection.

Previously, two studies suggested that surgical injection of plasmid directly into mouse spleen resulted in integration of plasmid into the cellular DNA. In the first study [19], an inability to amplify one region of the plasmid by PCR was interpreted as a breakpoint caused by integration. This interpretation would mean that every plasmid was integrated and that every integration had a breakpoint in the same region of the plasmid. The second study [20] used inverse PCR to try to amplify genomic-plasmid junctions. Failure to obtain a 15-kb amplification product was interpreted as lack of free plasmid, and a smear of PCR products was interpreted as representing integrated

DNA, although the PCR products were not characterized and technical artifacts could explain these results. Schubbert et al. [21] used λ DNA cloning to detect integration of M13 DNA in spleen cells following ingestion by mice. However, the linking of the M13 DNA to genomic DNA could have occurred during the cloning ligation step (one clone had M13, mouse, bacterial, and rearranged λ DNA linked together). None of the above studies provided quantitative information. While lymphocytes in the spleen may be more prone to integration than nondividing muscle cells, the frequency of integration, if it occurred at all, could not be determined from these studies.

In summary, we describe a quantitative assay for investigating integration of plasmid DNA in vivo. Our results suggest that all or virtually all of the plasmid that persists in mouse muscle after injection is extrachromosomal. If integration occurred at all, the frequency would be at least three orders of magnitude below the spontaneous mutation rate, indicating that the risk of mutation due to integration is negligible. Although the present study investigated intramuscular injection in mice, the assay is applicable to other systems [14]. An important extension of the integration study is to carry out a thorough tissue distribution study, investigating plasmid levels and potential integration at distal sites [see 14].

Acknowledgments

The authors thank Sangeetha Sagar, John Shiver, Donna Montgomery, Robert Evans and P.K. Tsai for the preparation and characterization of the plasmid DNA vaccines, Donna Montgomery for assistance with V1Jp-Hygro transfections, Steven Hall, Beth Muller, Brenda Givler, Donna Lynch, Kim Trick and Beth Jackson for assistance with animal studies, Tom Skopek for the worst-case comparison with spontaneous mutation rate, and Keith Soper for the statistical analysis of sensitivity loss due to *Sfi*I digestion.

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