PhD ÉRTEKEZÉS

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DISZTROFIN MOLEKULÁK EXPRESSZIÓJA MDX IZOMBAN

PhD értekezés

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KÖSZÖNETNYILVÁNÍTÁS

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TARTALOMJEGYZÉK

A génterápia célja a sejtekben folyó fehérjeszintézis minôségi és/vagy mennyiségi befolyásolása új gének bevitelével. A kérdéses gén hordozója lehet vírus, vagy egyszerû plazmid DNS (pDNS), melynek in vitro bejuttatására eukarióta sejtekbe különbözô fizikokémiai módszerek használatosak (Dankó és Wolff, 1994). Figyelemreméltó, hogy harántcsíkolt izomsejtek külön manipuláció nélkül is képesek pDNS-t az extracelluláris térbôl felvenni (Wolff, Malone és mtsai., 1990). Egér vázizomba injektált, riportergént tartalmazó plazmid bejut a sejtmagokba, és expressziója az állatok egész életére megmarad anélkül, hogy integrálódna a kromoszomákba (Dankó és Wolff, 1994, Wolff, Ludtke és mtsai., 1992). Az izomsejtek e különleges tulajdonsága lehetôvé teheti öröklött primér myopáthiák génterápiás korrigálását, és más felhasználási lehetôségeket is ígér, melyek közül említést érdemel vírusfehérjék expessziója immunizálás céljából, vagy a szervezet távolabbi pontjain ható fehérjék, mint növekedési hormon (Dhawan, Pan és mtsai., 1991) és IX alvadási faktor (Yao és Kurachi, 1992) termelése és szekréciója.

Munkánk során az egyik leggyakoribb herediter izomsorvadás, a *Duchenne Muscularis Dystrophia* (DMD) egy állatmodelljében tanulmányoztuk plazmid DNS-be épített normális és mutáns disztrofin cDNS expressziójának hatásait az izomrostokra. A DMD nemhez kötötten öröklôdik, kb. 1/3000 fiút érint. Jelenleg hatásos kezelése nincs, befolyásolhatatlanul progrediáló izomsorvadás jellemzi, ami 4-5 éves kor körül okozza az elsô tüneteket és fiatal felnôttkorra betegek halálához vezet (Papp, 1990, Robinson és Linden, 1993). A beteg izomrostokra jellemzô a *disztrofin* nevü 427 kD molekulasúlyú fehérje hiánya vagy aberrációja (Hoffman, Fishbeck és mtsai., 1988, Hoffman, Brown és mtsai., 1987).

A disztrofint kódoló gén melynek mutációja a kórkép hátterében áll, az X kromoszómán helyezkedik el, 79 exont foglal magába és több mint 2.5 millió bázispárra terjed ki (Koenig, Hoffman és mtsai., 1987). A génről különböző promoterek irányítása alatt többféle transzkript íródik (Ahn és Kunkel, 1993). Az elsősorban a váz- és szívizomban előforduló disztrofin izoformának, amely DMD-ben hiányzik vagy kóros, egy 14 kb nagyságú mRNS felel meg (Hoffman, Brown és mtsai., 1987). A gén hatalmas mérete miatt a mutációk gyakoriak és sokfélék, változatos kliniai képet eredményezve. Az enyhébb fenotípus Becker-, a súlyosabb Duchenne-féle izomdisztrófia néven ismert. A diagnózisra került esetek 1/3-a új mutáció következménye (Robinson és Linden, 1993), tehát genetikai vizsgálatokkal és tanácsadással a gyakorlatban nem előzhető meg, ami kiemeli a hatásos kezelés szükségességét.

A disztrofin molekula négy fő részre, (a) amino-terminális, (b) centrális, (c) u.n. ciszteinben gazdag és (d) karboxil-terminális doménekre osztható. A plazmalemma belső felszíne mentén elhelyezkedve komplexet alkot az amino-terminális domén felől az izomrost aktinjával, a karboxil-terminális részen keresztül pedig egy négy tagból álló transzmembrán glikoprotein komplexszel ("dystrophin-associated glycoproteins"-DAG) (Ervasti és Campbell, 1991). A DAG komplex a szarkolemma extracelluláris felszínén kapcsolódik az alfa-disztroglikán nevű glikoproteinhez, és azon keresztül az extracelluláris lamininhez. Ezáltal a disztrofin molekulán keresztül horgonyzódnak az izomrost kontraktilis fehérjéi a szarkolemmához és az extracelluláris matrix fehérjéihez (Ervasti és Campbell, 1993). Ismert, hogy a disztrofin-hiány a DAG komplex fehérjéinek drasztikus csökkenésével is jár (Ohlendieck, Matsumura és mtsai., 1993). Jelen elképzelés szerint DMD-ben az izomnekrózis annak a következménye, hogy megszűnik az összeköttetés a szubszarkolemmális citoszkeleton és az extracelluláris matrix között és ez esendőbbekké teszi a sejteket az izomkontrakciók okozta mechanikus stresszel szemben (Matsumura és Campbell, 1994).

A humán X-hez kötött izomdisztrófiának több állatmodellje ismert, géntherápiás kutatás céljára az *mdx egér* (Bulfield, Siller és mtsai., 1984) használatos a legszélesebb körben. Az eredeti mutáns izomsejtjeiben az X-kromoszómán lévő nonszensz pontmutáció

következtében nem termelôdik disztrofin (Sicinski, 1989). Különösen idôsebb mdx egerekben azonban, viszonylag nagy számban fordulnak elô disztrofin-pozitív "revertáns" rostok, melyek valószínûleg második, szomatikus mutáció eredményei (Hoffman, Morgan és mtsai., 1990). Ezek a disztrofin expresszió immunhisztokémiai kiértékelését nagyon megnehezítik. A disztrofin-géntranszfer eredményességének immunhisztokémiai tanulmányozására olyan állatmodellre van szükség, amelyben annyira alacsony a háttér revertáns rostok száma hogy viszonylag kis hatásfok mellett is biztonságosan értékelhetô a génexpresszió.

A DMD géntherápiájának sikere attól függ, hogy elérhetô-e megfelelô számú izomrost transzformációja, és hogy a génrexpresszió az adott sejtek fenotípusát normalizálja-e. A disztrofin strukturális fehérje, korlátozott nukleáris doménnel (Pavlath, Rich és mtsai., 1989), ezért sok sejtmagnak kell normális génkópiát tartalmaznia ahhoz, hogy az izomostok teljes kiterjedésében termelôdjön. Jelenleg a vírusvektorok tünnek alkalmasnak arra, hogy klinikailag is hasznosítható hatásfokkal transzformálják az izomrostokat, azonban a disztrofin kódszekvencia meghaladja befogadóképességüket.

Kísérleteinkkel ahhoz a munkához kívántunk hozzájárulni, melynek célja olyan *megfelelô therápiás transzgén* konstruálása amelynek expressziója megakadályozza az izomrostok pusztulását, és a nagyobb hatásfokú, de kisebb befogadóképességû adenovírus vektorokba is behelyezhetô. Ehhez elengedhetetlen a disztrofin molekula funkcionális szempontból nélkülözhetô részeinek meghatározása. Munkánkhoz plazmidvektorokat használtunk, melyek képesek a teljes cDNS-t befogadni és így alkalmasak különbözô disztrofin mutánsok funkcionalitásának összehasonlító vizsgálatára.

A disztrofin expresszió sajátosságait tanulmányozva etil-nitrozo-urea (ENU) expozícióval létrehozott új mdx variánsokban (Chapman, 1989), két új mutáns allélt hordozó törzset írtunk le (mdx4 és mdx5) (Dankó, Chapman és mtsai., 1992), melyek a revertáns rostok alacsony száma miatt disztrofin immunhisztokémiai vizsgálatok céljára kiválóan alkalmasak. Ezeket az egértörzseket használtuk kísérleteinkhez, melyekben disztrofin mutánsok expresszióját vizsgáltuk.

A disztrofin molekula egyes részei hiányának következményeit különböző deléciókat tartalmazó cDNS-ek expressziójával járó fenotípus változások összehasonlításával tanulmányoztuk. Az állatok quadriceps izmába rekombináns plazmidot injektáltunk, mely vagy a teljes cDNS-t vagy olyan disztrofin molekulákat kódoló mutáns cDNS-t tartalmazott, melyekből hiányzott a centrális domén nagy része (Becker) (England, Nicholson és mtsai., 1990), az amino-terminus és/vagy a karboxil-terminus (Fritz, Dankó és mtsai., 1995). A géntranszfert követően vizsgáltuk a disztrofin és a DAG komplex fehérjéinek kimutathatóságát a szarkolemma mentén, valamint a centrális elhelyezkédesû sejtmagok elôfordulási arányát. Centronukleált izomrostok korábbi degenerációs-regenerációs ciklusok következtében jelennek meg (Grounds, 1991), arányuk csökkenése a progresszió lassulását mutatja. A génexpresszió stabilitása a transzdukált izomrostok tartós túlélését jelzi, változását két módszerrel is tanulmányoztuk. Egyrészt immunhisztokémiai vizsgálatokkal nyomonkövettük a rekombináns disztrofint tartalmazó izomrostok számának alakulását, másrészt olyan markert alkalmaztunk, melynek expressziója párhuzamos a disztrofin expressziójával, de kimutatása a disztrofin kimutatásától független (Dankó, Fritz és mtsai., 1993, Fritz, Dankó és mtsai., 1995). Nevezetesen, olyan plazmidokat állítottunk össze, melyek a különbözô disztrofin cDNS-ek mellett a luciferáz gént

is tartalmazták. Luciferáz expresszió tehát csak olyan sejtmag működésének eredményeként lehetett kimutatható, amely disztrofin gént is tartalmazott. A luciferáz expressziót azért választottuk markerként, mert az normális izomban stabil, mdx egérben viszont hamar elvész az izomrostok pusztulása miatt.

(1) Két ENU mutagenezissel létrehozott új mutáns mdx törzset írtunk le, melyekben a vizsgálatok sokkal megbízhatóbban végezhetők a "háttér" disztrofin-pozitív rostok alacsony száma miatt. Egyéb tulajdonságokban, mint hisztopathológiai jegyek és emelkedett szérum kreatin kináz, az állatok megkülönböztethetetlenek voltak az eredeti mutánstól. Aktív nekrotikus gócok és az egyes sejtmagokban folyó DNS replikációt jelző brómdeoxiuridin beépülés és retrovírus-vektor expresszió az állatok egész élettartama során kimutathatók voltak. Az állatok életkorának előrehaladtával nőtt a rostok közötti kaliberingadozás és a centrális elhelyezkedésű sejtmagok aránya, ugyancsak jelezve az ismételten visszatérő degenerációs-regenerációs ciklusokat. A többi vizsgált vázizommal szemben, a rekeszizomban jelentős kötőszöveti felszaporodás is észlelhető volt. Az állatokat több kutatócsoportnak is rendelkezésére bocsátottuk.

(2) A szövettani vizsgálatok alapján a teljes és a Becker disztrofin expressziója legalább hat hónapig stabil volt és a centrális elhelyezkedesű magok aránya csökkent a kontrollokhoz viszonyítva, jelezve, hogy a transzformált rostok esendősége kisebb lett.

A többi mutáns expressziója csak egy hétig volt kimutatható a plazmid injekció után. A plazmamembrán mentén helyezkedtek el azok a rekombináns disztrofin proteinek is melyekből hiányzott az amino-terminus, karboxil-terminus, vagy a centrális domén nagy része. Csak a karboxil-terminussal rendelkező molekulák voltak képesek stabilizálni a DAG komplexet a sejtmembrán mentén, és csak ezeknek a molekuláknak az expressziója volt kimutatható egy héten túl.

(3) A génexpresszió megbízhatóbb értékelése céljából egy másik, új megközelítést is alkalmaztunk azzal, hogy olyan expressziós kazettákat építettünk a plazmidvektorokba melyek az adott disztrofin mutánson kívül luciferáz riportergént is tartalmaztak. Ezáltal az immunhisztokémiai vizsgálatoktól függetlenül, indirekt módon is értékelhető volt a disztrofin gén expressziójának stabilizáló hatása az izomrostokra.

Hasonlóan a szövettani vizsgálatok eredményeihez, csak a teljes és a Becker disztrofint (is) kódoló plazmidok injekciója után volt a luciferáz expresszió legalább két hónapon át stabil mdx izomban.

Tekintettel a plazmid vektorok elônyeire (változatlan cirkuláris formában tartósan kimutathatók a sejtmagban, a kromoszomákba nem integrálódnak, nem replikálódnak, nagyobb inszerteket képesek befogadni mint a jelenleg ismert vírusvektorok, nem vezetnek replikációra képes vírus keletkezéséhez, kisebb a velük kapcsolatos immunkomplikációk veszélye) a pDNS alapú géntranszfer fejlesztése továbbra is érdeklôdésünk középpontjában áll. Mint ismert, a klinikai alkalmazás fô akadálya az alacsony hatékonyság. Jelenleg folyó munkáinkban a pDNS expresszió fokozásának lehetôségeivel foglalkozunk. Vizsgáljuk az izomregeneráció, életkor, species, törzs, plazmidszerkezet (vektor, regulátor- és kódszekvenciák, intronok, stb.) hatását a génexpresszióra.

(1) Kimutattuk, hogy a recipiens izom pDNS injekció elôtti Bupivacaine (Marcaine) elôkezelésével akár 40-szeresére lehet növelni a luciferáz riportergén expresszióját (Dankó, Fritz és mtsai., 1994) A Bupivacaine egy amid helyi érzéstelenítô, amely izomnekrózist okoz miközben a szatellita sejteket, érendotheliumot és az extracelluláris matrixot megkíméli; alkalmazását teljes regeneráció követi és izomszövet krónikusan nem károsodik.

Megfigyeléseink azt mutatták, hogy az elôkezelés 5-7 nappal a plazmid injekció elött a leghatásosabb, tehát az expresszió akkor nô leginkább, ha a pDNS injekció intenzíven regenerálódó izomba történik. Az expresszió egy hónap után éri el a legmagasabb szintet, majd meredeken esik. Az elôkezelt izomban mért fokozott luciferáz aktivitás oka lehet fokozott pDNS felvétel a regenerálódó sejtek által, vagy fokozott expresszió transzkripcionális vagy transzlációs okok miatt. Az utóbbit látszik alátámasztani, az hogy az elôkezelt izmok PCR analízise során nem találtunk jelentôsen több pDNS-t a kontrollokhoz viszonyítva.

Megjegyzendô azonban, hogy a szemikvantitatív PCR nem elég érzékeny módszer ahhoz, hogy a fokozott plazmid felvétel lehetôségét ki lehessen zárni. Logikus magyarázatként kínálkozik a fokozott expresszió is, mely kapcsolatban lehet a foetalis és embryonális miozin nehézlánc expresszió változásaival a regenerálódó izomban. Mivel az expresszió fokozódása csak átmeneti volt, a módszertôl gyakorlati haszon inkább immunizációs protokollokban mint intrinsic izombetegségek kezelésében remélhetô.

(2) A optimális életkor a pDNS izomba juttatására a maximális génexpresszió szempontjából két hét volt, három különbözô egértörzsben (Balb/C, C57/Bl, ICR) és Sprague-Dawley patkányban, különbözô promoterekkel (CMV, RSV, SV40, MCK) és különbözô riportergénekkel (Luc, Lux, beta-galaktozidáz, Becker-Dystrophin))(Dankó, Williams és mtsai., 1997).

Valószínü, hogy a fokozott pDNS felvétel legalább részben szerepet játszik ebben, ugyanis fluoreszcens pDNS injekciója után 2 hetes állatokban kimutatható volt, hogy az injektált plazmid egyenletesebben oszlik el az izomban, és korán megjelenik a szarkoplazmában, ellentétben az idôsebb állatokkal, melyek izmai nagyobb mennyiségü extracelluláris matrixot tartalmaznak. Az extracellularis barrierek jelentôségét támasztja alá az is, hogy a genetikusan M-laminin deficiens felnôtt homozygóta 129/ReJ-Lama2^{dy} egerekben észlelt luciferáz expresszió magasabb mint a normál heterozygotákban. Ugyancsak érdekes, hogy az egyébként differenciált izomban legaktivabb MCK promoterrel is a fiatal állatokban volt a legmagasabb az expresszió.

A fokozott expresszió azonban a promoterek fokozott transzkripciós aktivitásának a következménye is lehet; elképzelhetô, hogy bizonyos, az alkalmazott promoterekre ható transzkripciós faktorok nagyobb mennyiségben vannak jelen fiatal izomban.

A plazmidok összetétele ugyancsak lényeges eleme volt a génexpressszió közötti különbségeknek, nevezetesen a pCI alapu plazmidok CMV promoter és a Luc gén eredményezték a legmagasabb értékekett.

A fiatal állatokban elért magas génexpresszió is átmenetinek bizonyult, valamennyi alkalmazott promoterrel, hasonlóan a regenerálódó izomban tapasztaltakhoz. A teljes injektált izomra számított luciferáz stabilizálódott egy szinten, mely megfelelt az idôsebb állatokban elért mennyiségnek, tekintet nélkül a kezdetben észlelt szintekre. Lehetséges, hogy a nagymennyiségû luciferázt termelô izomrostokat az immunrendszer eliminálja; elôzetes adatok azt mutatják, hogy FK506 immunszuppresszió meghosszabbítja a génexpresszió idôtartamát a két hetes állatokban.

Fiatal kutyákban és majmokban is jóval magasabb volt a riportergén expresszió mint idôsebb állatokban, a rágcsálókhoz hasonlóan magas értékeket azonban nem lehetett kimutatni. Magasabb rendû állatokban tehát egyenlôre nem tudtunk kielégítôen magas génexpressziót elérni, átmenetileg sem. További munkánk ennek elérését célozza.

Elôször vizsgáltuk különbözô amino- és karboxil terminális deléciót tartalmazó mesterséges disztrofin-mutánsok posztnatális géntranszfert követô expresszióját mdx izomban. Azt találtuk, hogy lehetséges a molekula membránlokalizációja az amino- és karboxil-terminus akár együttes hiányában, vagy nagy centrális domén deléció esetén is. Ennek alapján a disztrofin molekula több részén is képes kötôdni a plazmamembránhoz. Eredményeink azt támasztják alá, hogy a karboxil domén kritikus a DAG komlexszel való kötôdés szempontjából. A DAG komlexszel való kolokalizáció nem volt elôfeltétele a különbözô disztrofin molekulák membrán-lokalizációjának, de szükséges volt a mutáns disztrofin molekulák expressziójának stabilitásához, és a disztrofin hiány hisztopathológiai jeleinek enyhítéséhez.

Ezek az adatok egyrészt hasznos iránymutatók az optimális therápiás transzgén megtalálásához, másrészt bizonyítják, hogy a posztnatális géntranszfer hasznos lehet DMD-ben amennyiben elegendô számú izomrostban el lehet érni a teljes vagy Becker-disztrofin expresszióját.

Az eredmények klinikai hasznosításának legnagyobb akadálya a géntranszfer alacsony hatásfoka. Eddigi munkánk során csak átmenetileg és csak rágcsálók vázizmaiban sikerült magas génexpressziót elérnünk. További ismeretekre van szükség a harántcsíkolt izomba tuttatott pDNS expressziójának részletes mechanizmusait illetôen, hogy tovább tudjunk lépni. Jelenlegi kutatásaink a pDNS alapú géntranszfer hatásfokának növelését célozzák. Gének átvitele sejttenyészetben kritikus szerepet játszott a funkciójuk megfejtésében. Az általunk leírt rendszerben fiatal állatoknál elért nagyon magas génexpresszió lehetôvé teszi, hogy egereket használjunk sejttenyészethez hasonló módon gének és azok mutáns formáinak gyors funkcionális elemzésére.

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The Frequency of Revertants in mdx Mouse Genetic Models for Duchenne Muscular Dystrophy

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ABSTRACT. The mdx mouse has been used for the development of cellular and gene therapies for Duchenne muscular dystrophy. The relatively frequent occurrence of dystrophin-positive muscle cells called revertants has hampered these efforts by interfering with data interpretation. The mdx4^{cv} and mdx5^{cv} dystrophin mouse mutants have approximately 10-fold fewer revertants than the mdx mutant at both 2 and 6 mo. The mdx3^{cv} dystrophin mouse mutant may be a useful model for some types of human dystrophin deficiencies in which the levels of dystrophin are low but not completely absent. (*Pediatr Res* 32: 128–131, 1992)

Abbreviations

DMD, Duchenne muscular dystrophy

The mdx mouse containing a nonsense point mutation in the dystrophin gene is a genetic model for human DMD (1). The mouse has increased serum creatinine phosphate kinase levels and muscle pathology that includes necrosis, cellular infiltration, wide range of fiber size, and a large number of centrally nucleated fibers. In contrast to DMD, fibrosis and fatty deposits are not typical for mdx skeletal muscle. Nonetheless, the mdx mice have been helpful for increasing our understanding of DMD by elucidating the role of the dystrophin-associated glycoproteins (2) and the role of elevated myofiber calcium in its pathogenesis (3–5). An understanding of why the mdx mouse is different from human DMD may prove critical to the development of a successful therapy (6). A recent report indicates that mdx diaphragmatic muscle has pathology similar to human muscle (7).

New mutant alleles of mdx were recovered from female progeny of ethylnitrosourea-treated male mice that are designated mdx2^{cv}, mdx3^{cv}, and mdx4^{cv} (8). More recently, we have established a fourth allele, mdx5^{cv}. The location of these new mutations has been established relative to a restriction fragment length polymorphism in the central region of the gene, such that mdx2^{cv}, -3^{cv}, and -4^{cv} map to the 3′ region of dystrophin gene, whereas mdx5^{cv} maps to the 5′ region (Chapman VM, Miller DR, unpublished data). These new mdx mutants lacked dystrophin in skeletal muscle as assayed by immunofluroescence and immunoblots and had skeletal muscle pathology similar to the original mdx mice.

A small percentage of the skeletal and cardiac muscle cells in mdx mice and humans with DMD contain dystrophin immunoreactivity against the background of dystrophin-negative cells (9). The nature of the dystrophin-positive cells referred to as revertants has yet to be determined. Postulated mechanisms for the revertants include *1*) genomic deletion of the exon containing the point mutation, *2*) expression of a suppressor transfer RNA, and *3*) expression of dystrophin-related protein. The ability of the revertants in the mdx mice to be stained with several different antibodies that recognized all of the dystrophin domains suggested that the revertants were expressing the X chromosome-encoded dystrophin protein. The expression of a dystrophin-related protein was altered in mdx mice, but its pattern of expression was different from that of the revertants (10, 11).

The mdx mouse has played a critical role in the development of new cellular transplantation and gene therapies (12, 13). The number of dystrophin-positive muscle cells before and after the experimental maneuvers is useful for assessing the efficacy of these experimental therapies. The presence of the revertants complicates this assessment. This study found that the mdx4^{cv} and mdx5^{cv} mutants contained approximately 10-fold fewer revertants than the mdx mutants.

MATERIALS AND METHODS

Mouse strains. Mdx mice (C57BL/10 mdx/mdx) and control BALB/c mice were obtained either from Jackson Laboratories (Bar Harbor, ME) or from V. Chapman's laboratory, which obtained the mdx mice directly from G. Bulfield's laboratory. The mdx2^{cv}, mdx3^{cv}, mdx4^{cv}, and mdx5^{cv} mutants were initially bred to congenicity onto the C57BL/6Ros background for more than 10 generations (8) and were subsequently maintained in both the Buffalo and Madison laboratories.

Dystrophin assays. Immunoblots were performed as previously reported using the 6-10 antibody (13, 14). After staining for dystrophin, the blots were stained with india ink to determine the amount of myosin that was transferred in each lane. Immunofluorescence analysis was performed on 6-μm frozen sections as previously reported (13) using 1-2a antibody directed against the amino terminus (15), affinity-purified 30-kd antibody directed against the rod domain (16), 6-10 antibody directed against another part of the rod domain (13), and d10 antibody against the carboxyl terminal domain (9, 16, 17). The appropriate biotinylated secondary antibodies (Amersham, Des Plaines, IL) were used with Texas red streptavidin. Muscle sections were stained with hematoxylin and eosin for routine histologic examination (18). Nuclei were localized by staining with propidium iodide.

Statistical analysis was performed using *t* tests.

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RESULTS

Skeletal muscle. The quadriceps muscles of the mutants were analyzed by immunofluorescent staining for dystrophin. As previously reported (8), the vast majority of the skeletal myofibers on the cross-sections from the mdx2, mdx5, and mdx4 mutants lacked dystrophin immunoreactivity. Unexpected results in the mdx3 mutant will be presented below. As in the mdx muscles, the skeletal muscles in the mdx2, mdx5, and mdx4 mutants had a few revertant fibers with dystrophin immunoreactivity at the sarcolemma. The most sensitive antibody, the 6-10 antibody, was used to reduce the chance of missing revertants with low levels of dystrophin (14). A large muscle group such as the quadriceps was chosen because its high number of revertants would provide a critical assessment of the number of revertants in the animals (9). Four to six quadriceps muscles were analyzed for each mutant. The number of revertants was counted in 10–20 sections of the middle part of the quadriceps, which contains approximately 4000 myofibers. The number of revertants among the different sections of the same muscle varied by less than 15%.

Figure 1 compares the mean number of revertants per quadriceps cross-section in the mdx, mdx2, mdx4, and mdx5 mutants at 2 and 6 mo. At 2 and 6 mo, both the mdx4 and mdx5 mutants had significantly fewer revertants than the mdx and mdx2 mutants ($p < 0.05$). At 2 mo of age, the mdx4 and mdx5 mutants had approximately 5- to 12-fold fewer revertants than the mdx and mdx2 mutants. At 6 mo of age, the mdx4 and mdx5 mutants also had 8- to 18-fold fewer revertants than the mdx and mdx2 mutants. From 2 to 6 mo, the number of revertants significantly increased ($p < 0.05$) 6.2-fold in mdx, 7.4-fold in mdx2, 4.0-fold in mdx4, and 4.6-fold in mdx5 mutants. In other muscles, such as the gastrocnemius, soleus, and diaphragm, the mdx4 and mdx5 mutants also had less revertants than the mdx and mdx2 mutants. Serial sections of the revertants in the mdx, mdx2, mdx4, and mdx5 mutants that were stained with the 6-10 antibody were also stained to some degree with the 1–2a, 30-kd, and d10 antibodies.

Sections of the mdx3 quadriceps stained for dystrophin immunofluorescence with the 6-10 antibody revealed that all myofibers contained sarcolemmal, dystrophin immunofluorescence (Fig. 2*B*). However, the intensity of the dystrophin staining was less than that in normal mice (Fig. 2*A*), but greater than the levels in the nonrevertant fibers of the other mutants (Fig. 2*C*). An increased variability in the diameters of the myofibers from the mdx3 mice was also apparent. Other dystrophin antibodies (1–2a, 60-kd, and d10) yielded similar results for the mdx3 mice.

In the mdx, mdx2, mdx4, and mdx5 mutants, approximately 70–90% of the nuclei in both the dystrophin-positive and dystrophin-negative myofibers were centrally located at 6 mo ($n > 500$ for each group). Greater than 70% of the mdx3 myofibers also contained central nuclei. Less than 1% of the myofibers from normal mice had central nuclei.

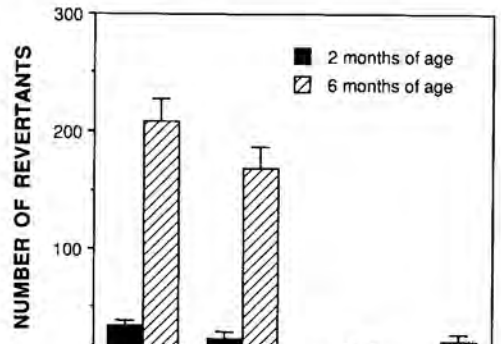

Fig. 1. The mean number of revertants in the mutants at 2 and 6 mo. The values represent the mean number of revertants in 10 to 20 sections of quadriceps muscles from four to six animals. *Bars* represent the SE.

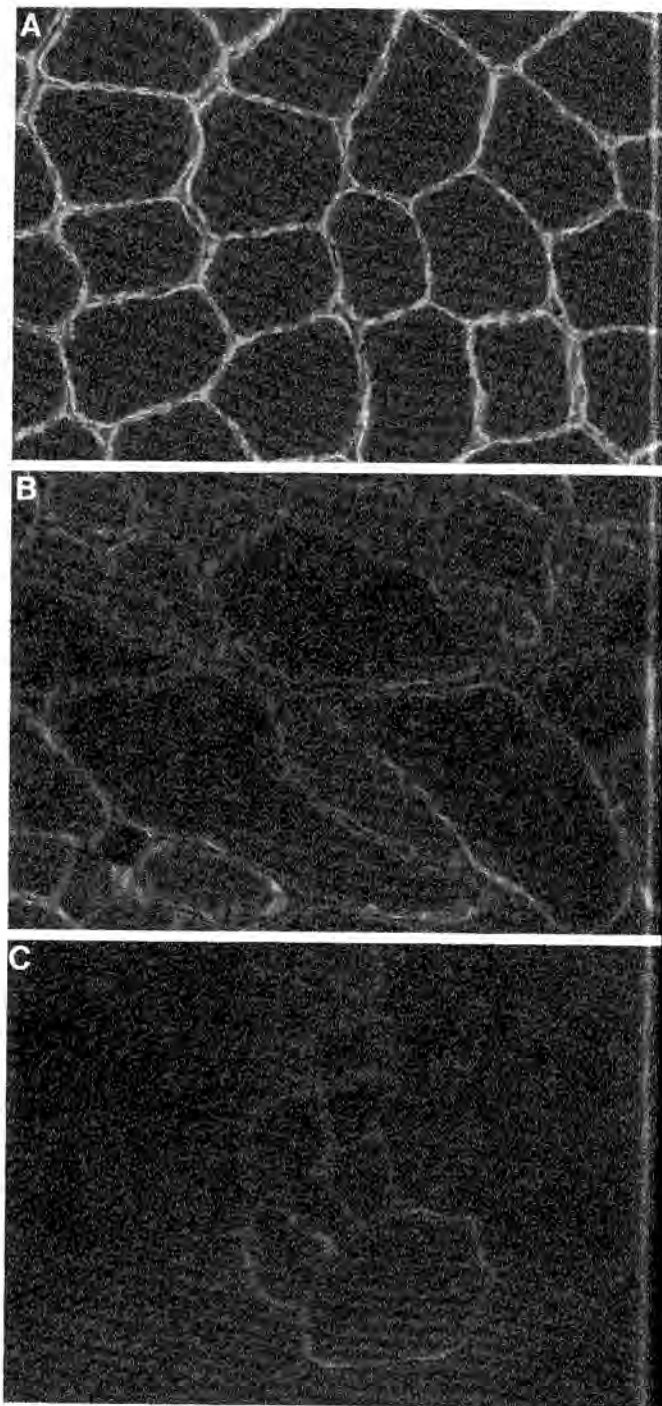

Fig. 2. Dystrophin immunofluorescence of 6-mo-old quadriceps muscles from a normal mouse (*A*), mdx3 mutant (*B*), and mdx4 mutant (*C*) showing a few revertants against a negative background. Magnification ×25.

The diaphragms of mdx mice have substantially increased fibrosis, fatty infiltration, and necrosis with cellular infiltration than the skeletal muscles of the extremities (7). The diaphragms of all four mutants contained amounts of fibrosis, fatty infiltration, and necrosis similar to those in the mdx muscle. The pathologic findings were more extensive with increasing age in all the mutants. As previously reported (8), the pathologic findings of the skeletal and cardiac muscles in the four new mdx mutants were similar to those in the original mdx mice.

Cardiac muscle. The number of revertants was evaluated in the myocardium of the mdx, mdx2, mdx4, and mdx5 mutants (Table 1). Revertants were also present in cardiac tissue from 6-mo-old mdx3 mutants because, unlike their skeletal muscle cells, all their cardiac cells did not have dystrophin immunofluorescence. At both 2 and 6 mo, the total number of revertants and clusters of revertants was greater in the mdx2 mutants than in the other mutants.

In the mdx, mdx2, and mdx4 mutants, the number of revertants was similar at 2 and 6 mo. Revertants were not observed in the cardiac muscles from the mdx5 and mdx3 mutants at 2 mo, but were observed at 6 mo. Given the difficulty in accurately determining the number of cardiac revertants, more hearts from the mdx3 and mdx5 mutants must be analyzed to determine whether the numbers actually increase over time in these mutants.

Immunoblot analysis of skeletal and cardiac muscles. Immunoblots using the 6-10 antibody were performed on quadriceps and cardiac muscles from normal, BALB/c mice and the five mutants (Fig. 3). After staining for dystrophin, the blots were stained with india ink to determine the approximate amount of protein transferred. Some of the lanes contained a band above dystrophin that most likely represents the cross-reaction of the 6-10 antibody with nebulin (13). No band above dystrophin in size was observed in the heart extracts because cardiac tissue does not contain nebulin.

Dystrophin was not observed in skeletal muscles from the mdx, mdx2, mdx4, or mdx5 mutants (Fig. 3*A*, *lanes 2, 3, 6,* and *7*), but low levels were observed as a faintly staining band in the mdx3 mutants (Fig. 3*A*, *lane 4*). These results were observed on more than 20 immunoblots on muscles from eight animals for each of the different mutants. Given the reproducibility of finding faint dystrophin staining in the mdx3 extracts and not in the other muscle extracts, it is unlikely that it was due to contamination.

Ten immunoblots were performed on extracts from five hearts for each of the mutants (Fig. 3*B*). Dystrophin was never observed in the mdx4 or mdx5 mutants (Fig. 3*B*, *lanes 1* and *2*), but low and variable amounts were observed in half of the 10 immunoblots performed on the extracts of mdx2 or mdx hearts (Fig. 3*B*, *lanes 6* and *7*). This variable and very faint dystrophin staining may have been observed only in this study to date because of the use of the sensitive 6-10 antibody. If it was due to expression of the X-chromosomal dystrophin or a dystrophin-related protein, it is not clear why it was only observed in the heart extracts.

In 10 immunoblots from five hearts of mdx3 mice, faint dystrophin staining was consistently observed to be greater than that in the mdx or mdx2 mice (Fig. 3*B*, *lane 4*).

Table 1. *Number of revertants in heart muscle of various mutants using 6–10 antibody*

Mutant	Number of revertants at 2 mo*			Number of revertants at 6 mo*		
mdx	1,1	2	1,2	2	2	1
	1	2	1,1	0	1	1
	1,3,1,1	1	1	1,2,2,2	1,3	2,2
	1,1	2	1	2,3	1,1	2,1
mdx2	1,2	1,1	1	1,1,1	3,6,2,1,1	1,1,1
	1	0	1	1	1	1
	1,6,1	5,1	1,1,4	1,2	1,1,1	2,1,1
	2,1	1,1,1	1,1,1	2,2	2,1,1	2,1,1
	3,1,1	1,1,3,5,3	1,2,6,2,2			
	8,1	6,2	2			
mdx3	0	0	0	1	3	1
	0	0	0	0	0	0
	0	0	0	0	0	0
	0	0	0	4	1,1	3,1
	0	0	0			
mdx4	0	0	0	1	1	1
	1	1	0	0	3	0
	1,2,2,1,4	4,1,2,2	2,2	1,1	2	1
	0	1,4	1	2,1	1	0
mdx5	0	0	0	1,1	1,1,1	1
	0	0	0	1,1	1,1,1,1	1,1
	0	0	0	1,3,3	1	1
	0	0	0	1	1	1,2

* The results are presented from three different sections, which contained the greatest number of revertants of the 10 sections examined. Each number represents the number of cells in each cluster of revertants. Each row is from a different animal.

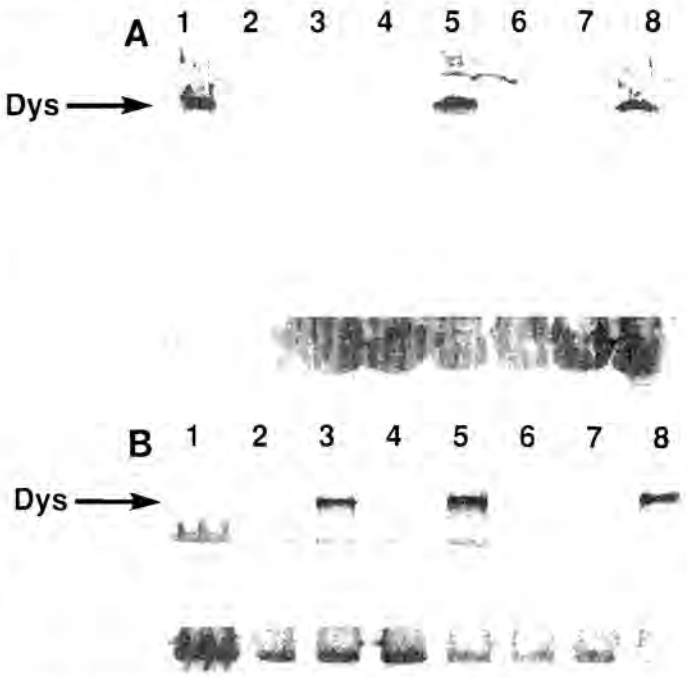

Fig. 3. Immunoblot analysis using the 6-10 antibody of quadriceps (*A*) and cardiac (*B*) muscles from the various mouse strains. *Lower panels* represent the myosin on the blots stained with india ink. *Arrows* indicate location of dystrophin. *A*, *Lanes 1*, *5*, and *8*, normal BALB/c; *lane 2*, mdx5 mutant; *lane 3*, mdx4 mutant; *lane 4*, mdx3 mutant; *lane 6*, mdx2 mutant; and *lane 7*, mdx. *B*, *Lane 1*, mdx5 mutant; *lane 2*, mdx4 mutant; *lanes 3*, *5*, and *8*, normal BALB/c; *lane 4*, mdx3 mutant; *lane 6*, mdx2 mutant; and *lane 7*, mdx.

DISCUSSION

The number of revertants in skeletal and cardiac muscle differed significantly among the mdx, mdx2, mdx3, mdx4, and mdx5 mutants. The mdx and mdx2 mutants both had the largest number of revertants with approximately 20 to 30 revertants per quadriceps cross-section at 2 mo of age, and approximately 200 revertants per quadriceps cross-section at 6 mo of age. The mdx4 and mdx5 mutants had approximately 10-fold fewer mutants than the mdx and mdx2 mutants at both 2 and 6 mo of age, with less than five revertants per quadriceps cross-section at 2 mo and less than 20 at 6 mo. The number of cardiac revertants was also greater in the mdx and mdx2 mutants than in the mdx5 or mdx3 mutants.

In the previous study, the number of revertants in mdx mice varied by at least 10-fold between the mdx mice maintained in the London and Boston laboratories (9). Different genetic backgrounds of the Boston and London mdx mice may have been the cause of their different reversion rates. Because the mdx2, mdx4, and mdx5 mice were bred to congenicity onto the C57BL/6Ros background for more than 10 generations, it is highly unlikely that their different reversion rates were due to different

genetic backgrounds. Viral infections may have also caused the disparity in revertant frequency between the mdx mice bred at the London and Boston laboratories. In this study, similar revertant frequencies were observed in the mice bred in either the Buffalo or Madison laboratory. Because the mutants were housed in the same room and analyzed in one laboratory, it is unlikely that the different reversion rates among the mutants were due to differences in viral infections or laboratory procedures. More likely, the reversion rates are different because of differences in the location or type of the point mutation in the various mutants. To further explore the basis for the different reversion rates among the mutants, mdx mice have been bred with mdx5 mice to generate female heterozygotes. The differences in reversion rates among the mutants has potential implications concerning the mechanism and nature of the revertants.

The relatively larger number of revertants in the mdx mutants has complicated the analyses of gene or cell therapies. The high background increases the difficulty in assessing the efficacy of the new therapies based on the ability of the experimental therapy to increase the number of dystrophin-positive fibers. The few revertants in the mdx4 and mdx5 mutants indicates that these mutants will be useful for the evaluation of gene and cell therapy approaches in mice. The pathology in the quadriceps, cardiac, and diaphragm muscles of the mdx4 and mdx5 mutants indicates that they will be as useful as the original mdx mouse in this regard.

The faint dystrophin immunofluorescence at the skeletal sarcolemma in the mdx3 mutants is of interest. None of the other mutants had such faint sarcolemmal immunofluorescence. On immunoblot analysis, the skeletal muscle of the mdx3 mutants also had small amounts of dystrophin, whereas the mdx, mdx2, mdx4, and mdx5 mutants did not. The low dystrophin levels in the mdx3 mutants were not able to prevent the pathology of the skeletal or diaphragm muscles. Human DMD patients with similar low levels of dystrophin have been described, and the mdx3 mutant will be useful as a model for this class of dystrophic patients (19, 20).

The hearts of the mdx3 mutants consistently had low levels of dystrophin on immunoblots, but their cardiac sarcolemma did not have dystrophin immunofluorescence. The sarcolemmal location of a mutated dystrophin in skeletal but not cardiac muscle has not been noted in humans previously, and its molecular basis is speculative. The mdx3 mutated dystrophin protein may be able to complex with dystrophin-associated proteins in skeletal muscle, but not in cardiac muscle (2), or perhaps the mdx3 dystrophin mRNA is spliced differently in cardiac and skeletal muscle (21).

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Dystrophin expression improves myofiber survival in *mdx* muscle following intramuscular plasmid DNA injection

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Expression of Becker-like and full-length human dystrophins was stable for at least 6 months in *mdx* mouse muscle following intramuscular plasmid DNA injection. Intramuscular injection of a single plasmid DNA encoding both luciferase and dystrophin resulted in stable luciferase expression for at least 2 months in *mdx* muscle, whereas injection of plasmid DNA encoding only luciferase did not result in stable luciferase expression. These results suggest that expression of either full-length or Becker-like dystrophins protects *mdx* mouse myofibers from degeneration.

INTRODUCTION

Duchenne muscular dystrophy (DMD) affects one in 3500 males with a progressive myopathy resulting in death (1, 2). Both DMD and Becker muscular dystrophy (BMD) result from a defective dystrophin gene (3). Gene therapy is a promising treatment for children affected with DMD. The two central issues concerning gene therapy for DMD are whether the dystrophin gene could be transferred and expressed in a sufficient number of myofibers, and whether dystrophin expression could prevent the progression of the disease state. One gene therapy approach for treating DMD involves intramuscular injection of dystrophin-encoding plasmid DNA. Skeletal and cardiac muscle have the ability to take up and express naked plasmid DNA injected into their extracellular spaces *in vivo* (4, 5). Injected plasmid DNA was stably expressed for at least 19 months in normal mouse muscle and persisted in an unintegrated, non-replicative, circular from (4, 6). The plasmid DNA may enter myofibers by an active uptake process that is specific to striated muscle (7). In *mdx* mice, a model for human DMD, approximately one percent of the myofibers expressed both Becker-like and full-length human dystrophins after intramuscular injection of dystrophin-encoding plasmid DNA (8). Both forms of dystrophin were expressed for at least one week following plasmid DNA injection (8). Although the percentage of myofibers expressing dystrophin was to low for this technique to be clinically applicable, intramuscular injection of plasmid DNA does provide sufficient dystrophin expression to study its ability to correct the DMD phenotype at the myofiber level. For example, those *mdx* myofibers that expressed dystrophin showed an increase in the proportion of peripherally located nuclei (8). Such examinations have been substantially improved by the identification of *mdx* mice which contain lower numbers of revertant fibers (9, 10) than the originally described mutant (11). While retroviral and adenoviral vectors can be used to transfer the Becker-like gene into post-natal *mdx* muscle (12, 13), only naked plasmid DNA injection has successfully transferred the full-length dystrophin. In addition, adenoviral vectors have resulted in stable gene expression only when introduced into neonatal mice (14). Transgenic mouse techniques are being used to compare the effect of different dystrophins on the *mdx* phenotype (15–17), but the results involving the embryonic transfer of dystrophin genes may not extrapolate to the situation of somatic gene therapy at later development periods. This study presents evidence that the post-natal transfer of either full-length or Becker-like dystrophin-encoding plasmid DNA resulted in long-term dystrophin expression and that dystrophin expression greatly slowed myofiber degeneration in *mdx* muscle.

RESULTS

Stability of human dystrophin expression in *mdx* mice

The stability of human dystrophin expression was determined in mdx^{4cv} and mdx^{5cv} mice following intramuscular injection of plasmid DNA expressing either the full-length (pRSVDy) or Becker-like dystrophins (pRSVDy-B). The number of dystrophin-positive fibers per muscle cross-section were similar at one week, two months and six months after injection of pRSVDy into *mdx* muscle (Figs. 1 and 2). In the pRSVDy-injected muscle, one percent of the myofibers of the approximately 4,000 quadriceps fibers expressed dystrophin. This was five- to ten-fold greater ($p < 0.01$) than the number of dystrophin-positive fibers examined in pUC19-injected control muscle (Fig. 2). Becker-like dystrophin was also expressed for at least six months following pRSVDy-B injection, but in fewer fibers than seen in muscle expressing the full-length dystrophin (Figs. 1 and 2). In the pRSVDy-B-injected *mdx* muscle, the number of dystrophin-positive fibers decreased significantly between one week and two months ($p < 0.02$) but remained stable between two and six months. While the majority of the expressed dystrophin localized to the sarcolemma, more sarcoplasmic staining was observed in

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pRSVDy-B-injected muscle one week after injection (Fig. 1B) than in pRSVDy-injected muscle (data not shown). Only sarcolemma staining was observed six months after either pRSVDy or pRSVDy-B injection (Fig. 1C and 1D).

Instability of luciferase expression in *mdx* muscle

Luciferase activity was assayed in the quadriceps muscles of *mdx* mice at two weeks and two months after intramuscular injection of 10 μg of pRSVL plasmid DNA (Fig. 3A). Two weeks after plasmid DNA injection, luciferase expression was observed in both young (4–6 weeks of age) and old (1 year of age) *mdx* mice. These levels were similar to those obtained in normal mice (data not shown), although the luciferase activity measured in older mice was nearly half of that obtained in younger animals (Fig. 3A). By two months after plasmid injection, mean luciferase levels decreased by 24-fold ($p=0.0026$) and 14-fold ($p=0.0059$)

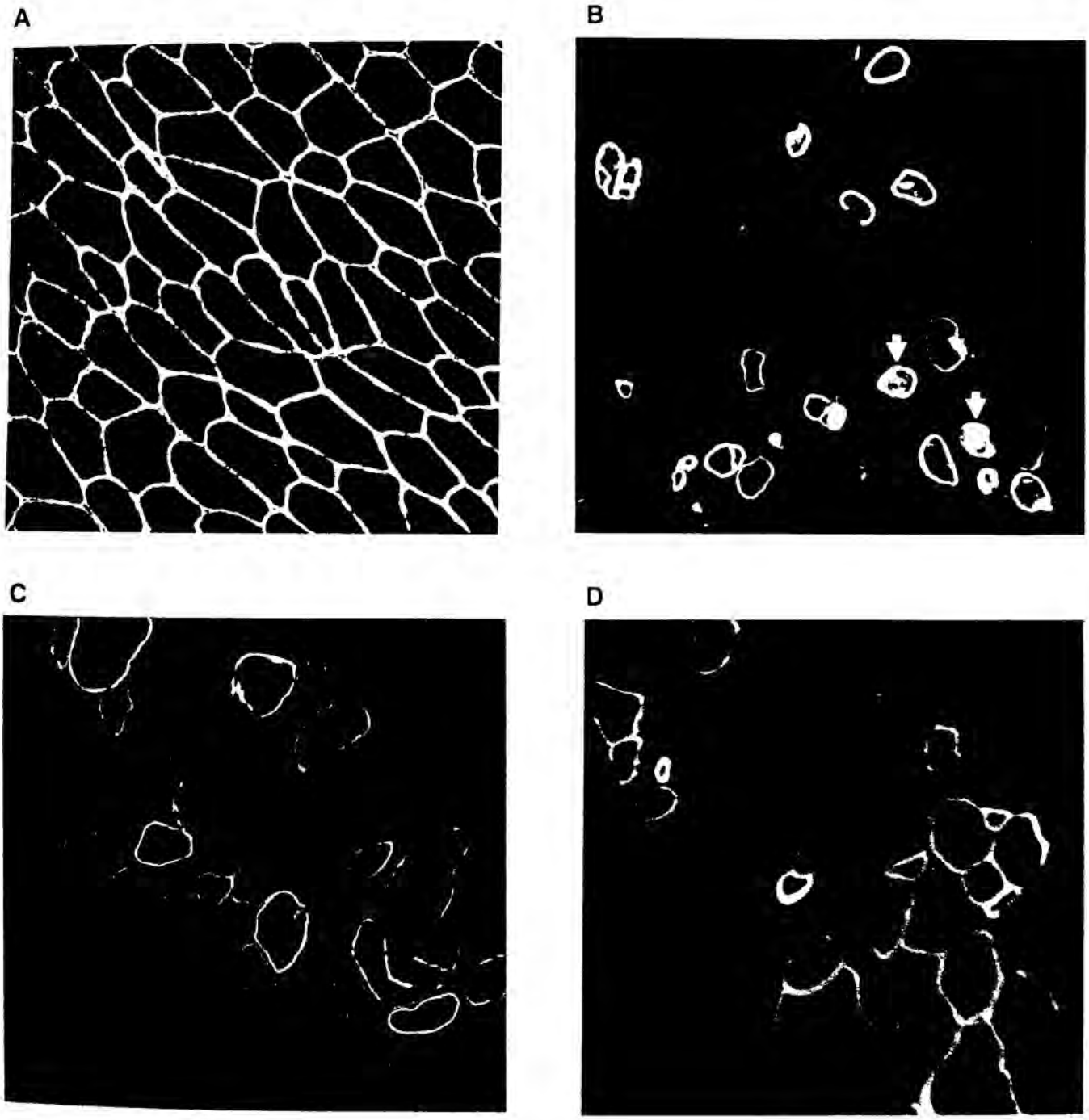

Figure 1. Immunohistochemical staining of dystrophin in quadriceps muscle of (**A**) normal Balb c mice, (**B**) *mdx* mice one week after pRSVDy-B intramuscular injection (closed arrows indicate fibers with cytoplasmic staining), (**C**) *mdx* mice six months after pRSVDy-B intramuscular injection, and (**D**) *mdx* mice six months after pRSVDy intramuscular injection. In (B), arrows indicate myofibers with sarcoplasmic dystrophin staining. The *mdx* mice were injected with 400 μg of plasmid DNA. Representative fields are shown at the site of plasmid DNA injection. Magnifications: 100× in A, C, and D and 40× in B (figures reduced to 70%).

in the young and old *mdx* mice, respectively (Fig. 3A). The luciferase activity measured at two months after plasmid DNA injection was not significantly different between young and old mice (Fig. 3A).

Luciferase expression was also unstable using two additional expression constructs that expressed luciferase from the SV40 promoter (pSV40L.1 and pSV40L.2) (Fig. 3B). These data suggest that luciferase expression was not stable in *mdx* muscle regardless of promoter or the animals' age.

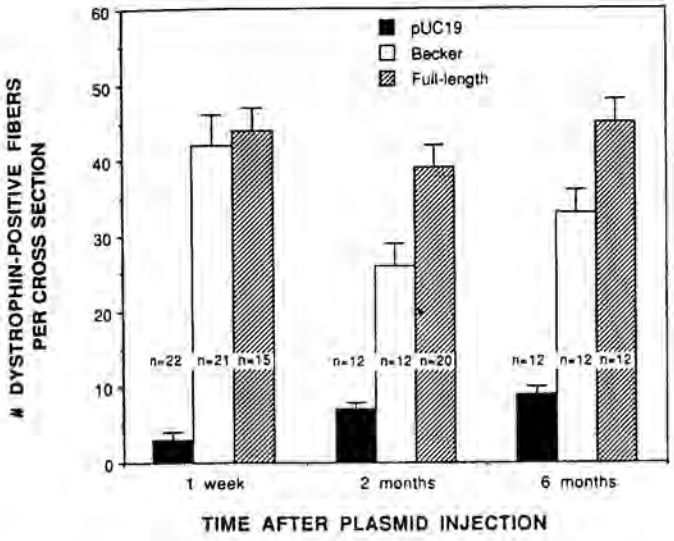

Figure 2. Stability of dystrophin expression in *mdx* mouse quadriceps muscle. Mice, 4–6 weeks of age, were given intramuscular injections of 100 μg of pUC19 plasmid as control (pUC19), 100 μg Becker-like expression plasmid (Becker), or 100 μg full-length dystrophin expression plasmid (Full-length). Mice were assayed for dystrophin expression at 1 week, 2 months and 6 months following plasmid injection. The total number of different quadriceps muscles examined for each treatment group is shown on the graph (n). Values represent the mean number of dystrophin-positive fibers observed per muscle at the site of plasmid DNA injection. Bars indicate standard error.

Stable co-expression of luciferase and dystrophin

Two plasmids were constructed to determine if co-expression of dystrophin and luciferase would impart long-term expression of luciferase to injected muscles (Fig. 4). Approximately 35 dystrophin-positive myofibers per cross-section were observed one week and two months after intramuscular injection of either plasmid into the quadriceps muscle of 6 week old *mdx* mice (data not shown). The luciferase activity measured at two months after plasmid DNA injection did not decrease from levels measured at two weeks in muscle injected with either co-expression construct (Fig. 5). Intramuscular injection of these co-expression plasmids appears to result in stable luciferase expression in *mdx* muscle for at least two months, although co-expression of Becker-like dystrophin resulted in higher luciferase activity than that obtained with full-length dystrophin (Fig. 5).

Degeneration and regeneration studies in old *mdx* mouse muscle

We have previously shown that luciferase expression after injection of luciferase-encoding plasmid DNA was stable in normal muscle (6). We hypothesized that the loss of luciferase expression in *mdx* muscle was due to dystrophin-deficiency-induced degeneration of myofibers containing plasmid DNA, and that this degeneration occurred regardless of the animals' age. To confirm this hypothesis *mdx* mice were injected with 5-bromo-2'-deoxyuridine (BrdU) to measure regeneration and degeneration (18). Quadriceps muscle from one year old *mdx* mice examined one day after BrdU treatment showed BrdU-labeled nuclei located predominately at the myofiber periphery (Fig. 6A). Two months after BrdU treatment, the number of peripherally located BrdU-positive nuclei had markedly decreased and many nuclei were located centrally in the myofibers of one year old *mdx* mice (Fig. 6B). Similar results were also observed in *mdx* mice 6 weeks of age at the time of BrdU injection (data

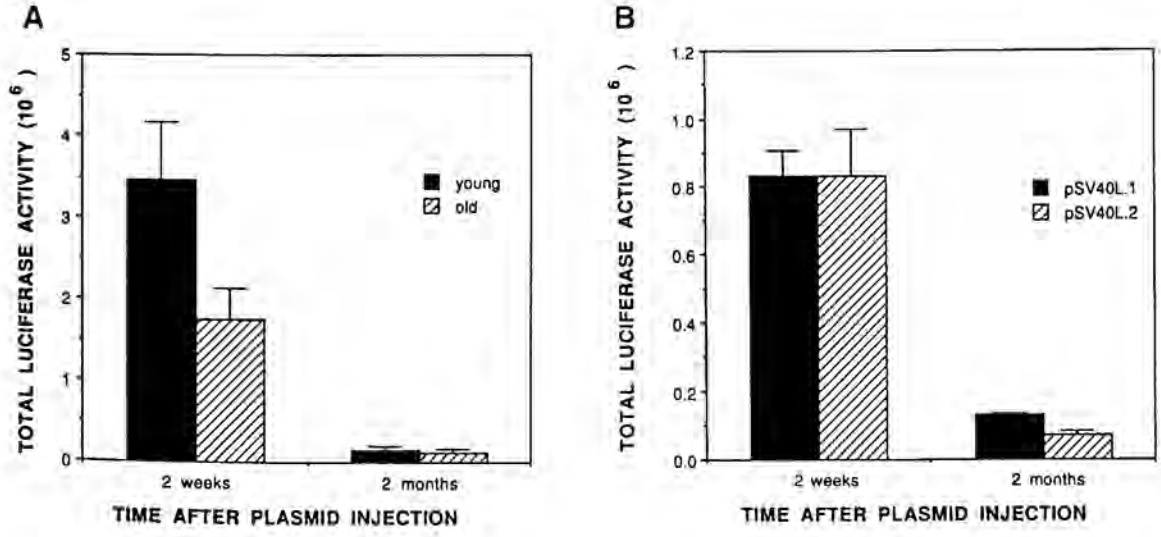

Figure 3. Stability of luciferase expression in *mdx* mouse muscle. (A) Mice, either 4–6 weeks (young) or one year (old) of age, were given intramuscular injections of 10 μg of pRSVL plasmid DNA. (B) Mice, 6 months of age were injected with 10 μg of either pSV40L.1 or pSV40L.2. Luciferase activity (in light units per entire quadriceps muscle) was determined for 8 different quadriceps muscles (n = 8) at 2 weeks and 2 months following plasmid DNA injection. Values represent the mean luciferase activity per treatment group. Bars indicate standard error.

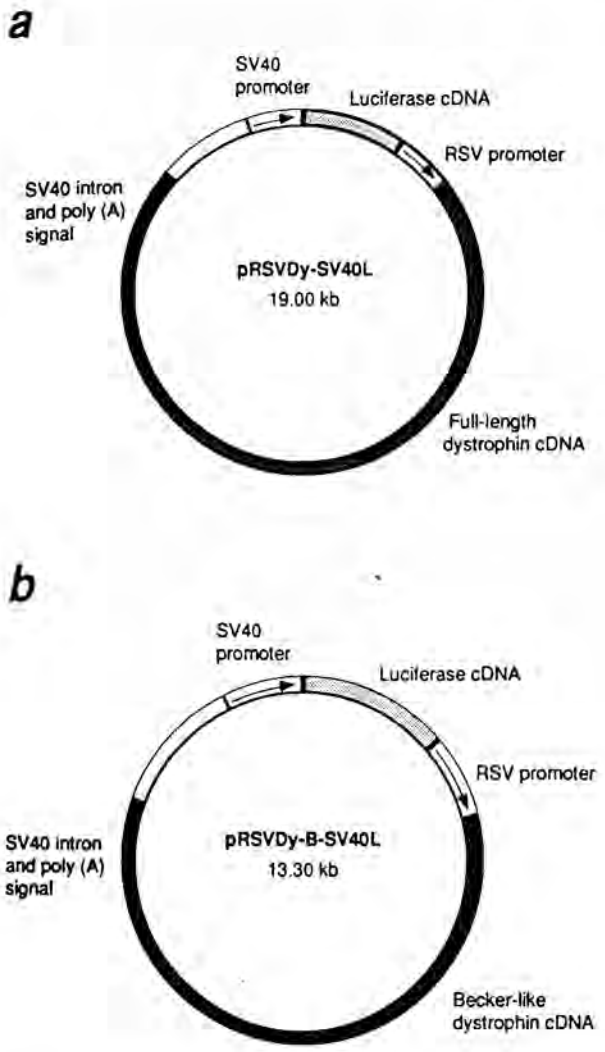

Figure 4. Diagram of the expression plasmid encoding cDNAs for both luciferase and full-length dystrophin (**a**), or luciferase and Becker-like dystrophin (**b**). Dystrophin expression is accomplished by the RSV promoter; luciferase by the SV40 promoter.

not shown). No BrdU-labeled nuclei were observed in normal quadriceps muscle of Balb/c, C57 or ICR mice (Fig. 6C).

β-galactosidase expression was observed in 2, 6 and 11 month old *mdx* muscle following the injection of replication-defective recombinant retroviruses containing the β-galactosidase gene (JR-gal) (Fig. 7). No β-galactosidase expressing myofibers were observed in Balb/c muscle (data not shown) unless it was treated with bupivacaine to induce widespread regeneration prior to retrovirus injection (Fig. 7).

DISCUSSION

This study explored the stability of dystrophin and luciferase expression in the *mdx* mouse model. From the data it was inferred that dystrophin expression slowed myofiber degeneration, one of the pivotal pathologic processes in DMD. It took advantage of the observation that luciferase expression was unstable in *mdx* muscle (Fig. 3) but was stable in normal muscle (6) after plasmid DNA injection. Luciferase expression, after pRSVL injection,

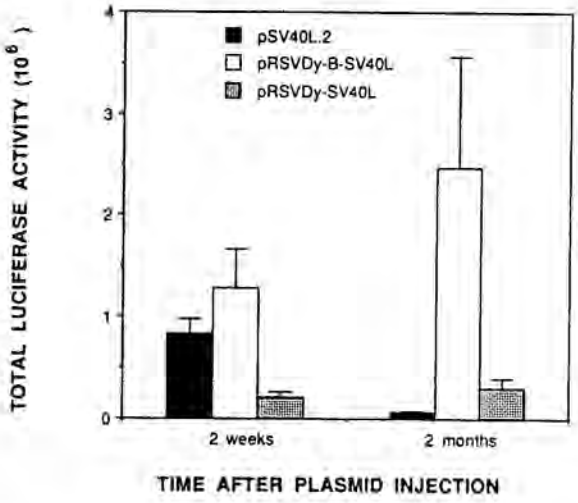

Figure 5. Stability of luciferase (Lux) expression in *mdx* mouse muscle. Mice, 4–6 weeks of age, were given intramuscular injections of 10 μg of pSV40L.2, 50 μg of pRSVDy-SV40L plasmid or 50 μg of pRSVDy-B-SV40L plasmid. Mice were assayed for luciferase expression at 2 weeks and 2 months following plasmid injection. Twelve quadriceps muscles were examined for each treatment group. Values represent the mean luciferase activity per treatment group. Bars indicate standard error.

was also unstable in cyclosporine treated mdx mice (unpublished data). Presumably, luciferase expression was unstable in *mdx* muscle because the plasmid DNA was lost as myofibers degenerated. Co-expression of either Becker-like or full-length dystrophin and luciferase from a single plasmid resulted in stable expression of luciferase for at least two months in *mdx* muscle (Fig. 5). It was implied from these results that dystrophin expression slowed myofiber degeneration in *mdx* muscle.

Co-expression of either Becker-like or full-length dystrophin and luciferase from a single plasmid was done to determine if those myofibers which expressed dystrophin were less susceptible to degeneration (Fig. 5). Five times more of the co-expression plasmids were injected into *mdx* muscle than pSV40L.2 so that approximately equal copies of the luciferase gene were injected into the muscles. The reason for the greater luciferase activity in muscle co-expressing the Becker-like dystrophin and luciferase compared to the full-length dystrophin and luciferase was unclear (Fig. 5). The greater luciferase expression observed in muscle injected with pRSVDy-B-SV40L than with pRSVDy-SV40L maybe due in part to the smaller size of pRSVDy-B-SV40L. Since the luciferase activity was stable in muscle injected with either co-expression vector, dystrophin expression appeared to stabilize luciferase expression. These results suggest that intramuscular injection of dystrophin-encoding plasmid DNA can provide, at the myofiber level, long-term dystrophin expression which in turn greatly slows myofiber degeneration. This result combined with the ability for post-natal dystrophin gene transfer in *mdx* mice to increase the percentage of peripherally-located nuclei (8) suggests that post-natal gene therapy may be efficacious in human DMD. Pre-natal dystrophin gene transfer in *mdx* mice has shown that over expression of dystrophin has no deleterious effects on the muscle, can result in near normal muscle function and morphology, and can restore dystrophin-associated glycoprotein expression (15–17).

The ability of both full-length and Becker-like dystrophins to slow degeneration in *mdx* myofibers has important implications

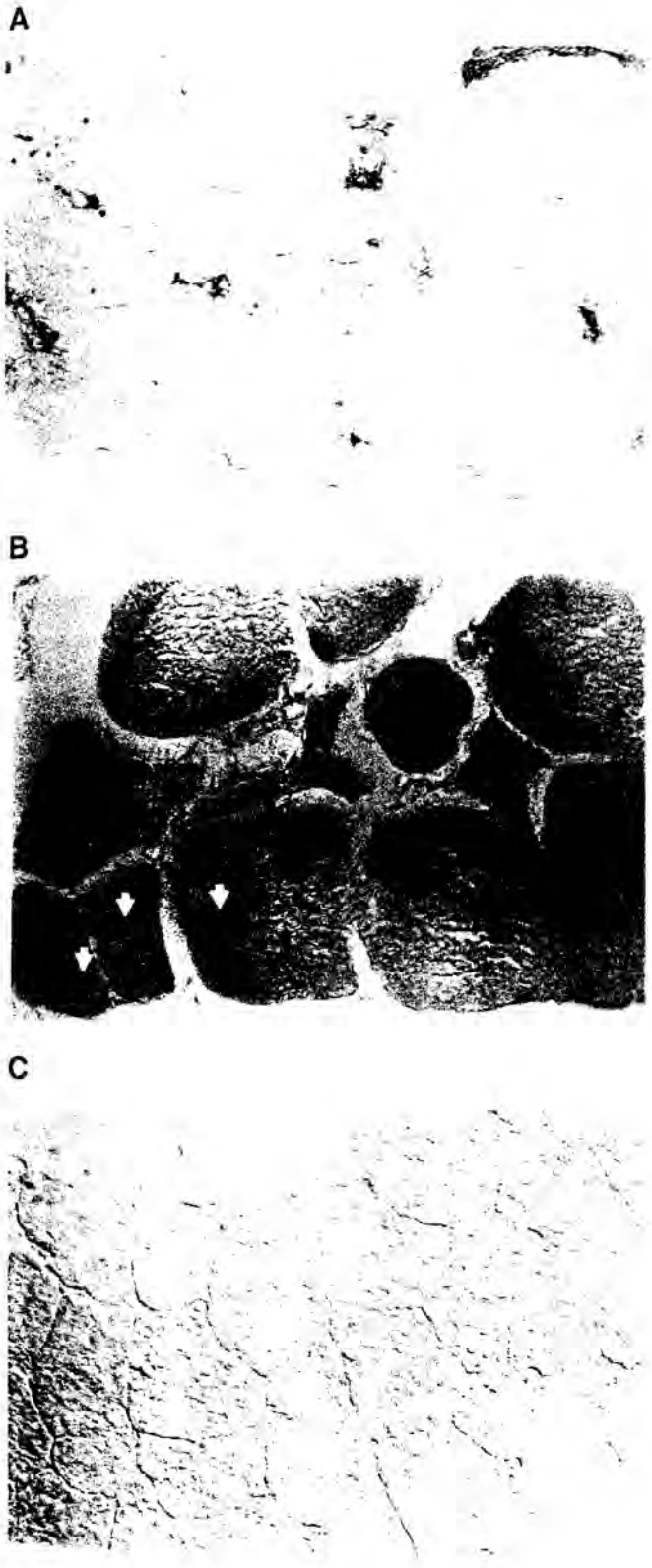

Figure 6. Photomicrographs of BrdU-labeled myonuclei in one year old *mdx* mice treated with BrdU. Representative fields are shown for *mdx* mice examined one day (**A**) and two months (**B**) after BrdU treatment, and for 6 week old normal mice examined one day after BrdU treatment (**C**). In (A) open arrows indicate non-labeled peripheral nuclei, arrowheads indicate non-labeled central nuclei, and closed arrows indicate labeled peripheral nuclei. In (B) closed arrows indicate labeled central nuclei. In (C) open arrows indicate non-labeled peripheral nuclei. Magnification, 100× (figures reduced to 52%).

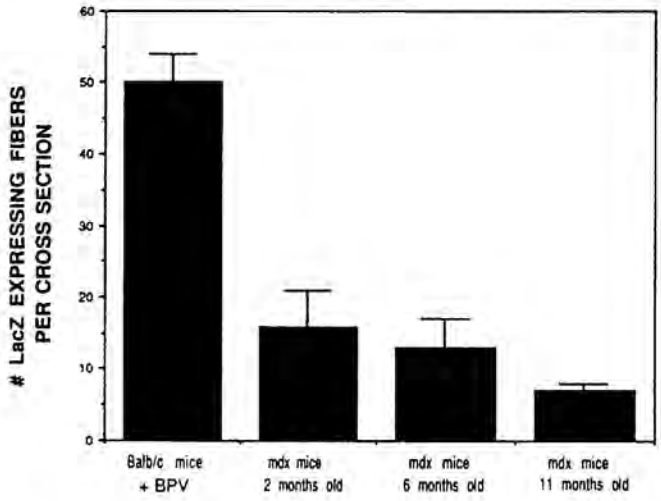

Figure 7. The number of β-galactosidase-positive myofibers in mouse quadriceps muscle after injection of a retrovirus containing the β-galactosidase gene. Balb/c mice were pre-treated with bupivacaine (BPV) three days prior to retroviral injection ('Balb/c mice + BPV'). All *mdx* mice were not pre-treated with bupivacaine and were 2, 6, and 11 months of age at time of retrovirus injection. Values represent the mean number of β-galactosidase (LacZ) expressing myofibers per muscle cross-section at the site of viral injection. Three muscles were analyzed for each group. Bars indicate standard error.

for future gene therapy efforts. Currently available viral vectors, such as retroviral or adenoviral vectors, can only accommodate the Becker-like dystrophin gene. Further development of these viral vector or other vector systems to transfer the Becker-like dystrophin gene into a clinically-relevant number of myofibers appear warranted by our results. However, further studies are required to determine whether the results in *mdx* mice extend to the *xmd* dog model or human DMD.

The instability of luciferase expression in both young and older *mdx* mice suggests that muscle degeneration continued as the *mdx* mice aged. A previous study using labeled nucleotides indicates that regeneration occurs in older *mdx* mice but at lower rates than young *mdx* mice (19). The incorporation of BrdU into the nuclei of old *mdx* muscle suggests that muscle regeneration continues as *mdx* mice age (Fig. 6A), while the loss of BrdU-labeled nuclei over time indicates myofiber turnover and suggests that old *mdx* muscle also underwent degeneration (Fig. 6). These labeled nuclei were predominantly associated with intact myofibers are were thus assumed to be myogenic nuclei. The presence of BrdU-labeled nuclei located centrally within myofibers (Fig. 6B) clearly indicates that some of the nuclei initially BrdU-labeled nuclei were myogenic. It also convincingly demonstrates that regeneration was occurring in muscle of one year old *mdx* mice. The ability of muscle from 11 month old *mdx* mice to express β-galactosidase following the injection of replication-defective recombinant retroviruses containing the β-galactosidase gene also suggests that muscle regeneration occurred in old *mdx* mice (Fig. 7).

Several histologic studies suggested that *mdx* myofibers underwent a major or perhaps even a single necrotic episode at three to four weeks of age from which they recovered to form myofibers with centrally located nuclei (20–22). More extensive histologic studies observed that young mice had the greatest muscle necrosis but fiber necrosis and regeneration in *mdx* mice

as old as 303 days was also observed (23–26). The electron microscopic appearance of Z-line streaming, an early indication of degeneration, in central nucleated myofibers suggested that such fibers were not protected from degeneration (23). The continued elevation of serum pyruvate kinase and creatine kinase in older *mdx* mice suggested that their sarcolemma remained abnormal (25).

Two recent studies using molecular techniques have come to opposite conclusions about the extent of degeneration/regeneration in older *mdx* mice. One study, using the expression of MyoD and myogenin as an index of regeneration, found elevated levels of MyoD and myogenin on Northern blots and by *in situ* hybridization in muscle of one year old *mdx* mice (26). Another study, using the expression of embryonic myosin heavy chain as a marker for regeneration, found that ten percent of myofibers contained embryonic myosin heavy chain in ten week old *mdx* mice but only one percent of the myofibers in either one year *mdx* mice or normal mice contained embryonic myosin heavy chain (27). The study also found elevated amounts of embryonic and neonatal myosin heavy chain mRNA on Northern blots in muscle of 16 week old *mdx* mice but the mRNA data in *mdx* mice older than 16 weeks of age was unfortunately not done.

The basis for the different conclusions concerning muscle degeneration in older *mdx* mice among the studies remains unclear but may reflect differences among colonies and the experimental methods. Perhaps the disagreement can be explained by the observation that regeneration in three to six week old *mdx* mice is more vigorous and segmental than that observed in mice of any other age. Thus, some of the experimental methods may not detect the more diffuse and less vigorous regeneration which occurred in older *mdx* mice. It is also possible, but unlikely, that the extent of degeneration and regeneration in the mdx^{4cv} and mdx^{5cv} strains used in this study differs from that in the original *mdx* strain. We have also observed unstable luciferase expression in the original *mdx* strain (data not shown).

In summary, the persistent *mdx* myofiber degeneration, that was inferred by three independent methods (unstable luciferase expression, BrdU-labeling, and retroviral transduction), was slowed by either Becker-like or full-length dystrophin expression in both young and old *mdx* mice.

MATERIALS AND METHODS

Mouse strains

The C57BL/6, Balb/c, and ICR strains were obtained from Harlan Sprague Dawley (Indianapolis, IN). The mdx^{4cv} and mdx^{5cv} strains have been previously referred to as the 2019 and 2222 mutants (9, 10). Given the indistinguishable phenotype of the mdx^{4cv} and mdx^{5cv} strains, they were used interchangeably and are simply referred to as *mdx* mice unless otherwise indicated.

Plasmid construction, preparation and injection

The pRSVL plasmid expresses the firefly luciferase cDNA (28) from the RSV promoter.

The plasmid pSV40L.1 was constructed as follows. The SpeI restriction site in the multiple cloning site (MCS) of pcDNA I/Amp (Invitrogen) was removed by partial digestion, filled in with Klenow, and re-ligated. The remaining SpeI site in this modified pcDNA I/Amp and the BamHI site in the MCS was used to remove the CMV promoter so that a SpeI/HindIII fragment containing the SV40 early promoter and a HindIII/BamHI fragment containing the luciferase cDNA (originally from pJD206) could be joined.

The pSV40L.2 plasmid was constructed as follows. The plasmid pBluescript SK(−) (Stratagene) was digested with PvuII to remove the entire MCS and ligated to the 1812-bp NruI/ScaI fragment from pcDNA I (Invitrogen) containing the CMV promoter, MCS, and SV40 polyA and splice site. The clone in which the CMV promoter was oriented adjacent to the ColE1 ori from pBluescript SK(−) was chosen and named pBlueCMV. The CMV promoter in pBlueCMV was removed by digesting pBlueCMV with NdeI/HindIII and replaced with the NdeI/HindIII fragment from pRSVL containing the entire RSV promoter to form pBlueCERSV. The HindIII/BamHI fragment from pJD206 containing the luciferase cDNA was inserted into the MCS of pBlueCERSV to form pBlueCERSVLux. The CMV/RSV fusion promoter in pBlueCERSVLux was removed by NruI/HindIII digestion and replaced with a SpeI (Klenow)/HindIII fragment containing the SV40 early promoter.

The plasmid pRSVDy-SV40L was constructed by ligating the SpeI/XhoI fragment from pSV40L.1 containing the SV40 promoter and luciferase cDNA into the NruI site in pRSVDy (just 5' to the RSV promoter). Similarly, the plasmid pRSVDy-B-SV40L was constructed by ligating the SpeI/Klenow fragment from pSV40L.2 containing the SV40 promoter and luciferase cDNA into the NruI site in pRSVDy. The clones that would transcribe from the SV40 promoter in the same direction as RSV promoter were chosen so that both transcripts would use the same splice and polyA sites.

All plasmids were purified by alkaline lysis and two CsCl gradients as previously described (29). Plasmid DNA injections into mouse quadriceps were done in 100 μl of normal saline (30). Specific amounts of plasmid DNA injected per quadriceps are given in the figure legends.

Dystrophin, luciferase and β-galactosidase assays

Immunofluorescence visualization of dystrophin-positive fibers was performed using rabbit antibody 6–10 which recognizes the rod domain of dystrophin (31) (generously provided by L. Kunkel), biotin-conjugated sheep anti-rabbit secondary antibody (Amersham) and streptavidin-Texas Red (BRL) (8). Determination of luciferase and β-galactosidase expression were done using previously described methods (4, 30).

BrdU injection and visualization

C57BL/6, Balb/c, and ICR and *mdx* mice were given two intraperitoneal injection of 5-bromo-2'-deoxyuridine (BrdU; Sigma Chemical Co., St. Louis, MO) at a dosage of 100 μg/gram body weight at 0 and 48 hours. At one day or two months following the last BrdU injection, the quadriceps muscles were removed from the mice and frozen in liquid nitrogen cooled isopentane. Frozen sections (8 μm) were fixed in 4% paraformaldehyde which had been treated with 70 mN NaOH. After fixation the sections were incubated with anti-BrdU antibody (1:20; Becton Dickson, Mountain View, CA) according to manufacturer's recommendations. After washing, the sections were incubated for 1 hour in peroxidase-conjugated goat anti-mouse secondary antibody (1:50; Hyclone, Logan, UT). Reactive nuclei were visualized by incubation in diaminobenzidine. The sections were examined using a Leitz microscope with conventional and Nomarski optics.

Retroviral transduction

Mice were injected with 10^5 amphotropic JR-gal retroviruses in 100 μl of DMEM culture medium (32). The JR-gal virus producing PA317 cell line was a gift of M.N. Gould, University of Wisconsin, Madison, WI. In this viral construct, the LacZ gene is expressed under control of the Moloney murine leukemia virus LTR and the neomycin-resistance gene is expressed from a SV40 early promoter (32). Virus titers were determined by infecting 3T3 cells with serially diluted virus stocks in the presence of 8 μg/ml polybrene (Sigma), followed by staining for β-galactosidase activity. Viral stocks were stored at −80°C. Normal mouse muscle was pre-treated by injecting 100 μl of 0.75% bupivacaine hydrochloride (BPV) (Abbott Laboratories) three days prior to retrovirus injection.

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Direct gene transfer into muscle

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Gene therapy has great promise for the treatment and the prevention of a broad range of inherited and acquired diseases. Gene transfer methods currently explored include the use of viral vectors and physical–chemical methods. Plasmid DNA can be taken up by skeletal muscle cells in vivo *without any special delivery mechanism and persist long-term in an extrachromosomal, non-replicative circular form. Thus, foreign genes can be expressed permanently in skeletal muscle. At present the efficiency of gene transfer is not high enough to treat genetic muscle diseases. However, even at the relatively low efficiency of expression we are able to achieve at present, plasmid DNA transfer seems to be a very promising way of programming cells* in vivo *to secrete proteins for immunization purposes.*

Keywords: Plasmid DNA; gene transfer; gene expression; gene therapy; luciferase; β-galactosidase; vaccination

Although the initial motivation for gene therapy was the treatment of genetic disorders, it is becoming increasingly apparent that gene therapy will be useful for the treatment and prevention of a broad range of acquired diseases, such as cancer and infectious disorders. Advances in our understanding of how genes are expressed during development and in a tissue-specific manner have allowed the expression of many protein-coding sequences in mammalian cells. Nonetheless, gene or polynucleotide transfer is the cardinal process of gene therapy. The gene needs to be transferred across the cell membrane and enter the nucleus where it can be transcribed. Gene transfer methods currently being explored include the use of viral vectors and physical–chemical methods. Viruses can be modified to carry a desired gene and become vectors for gene therapy. Using standard recombinant techniques, harmful or superfluous viral genes can be removed and replaced by the desired gene. This was first accomplished with mouse retroviruses[1]. The development of retroviral vectors was the catalyst that promoted current gene therapy efforts. Other viral vectors based on herpesvirus and adenovirus are also being developed.

Besides using viruses, it is possible to transfer genes into mammalian cells directly. Usually the desired gene is placed within bacterial plasmid DNA along with a mammalian promoter, enhancer and other sequences that enable the gene to be expressed in mammalian cells. The plasmid DNA can be incorporated into lipid vesicles (liposomes including cationic lipids such as lipofectin), that then transfer the plasmid DNA into the target cell. Plasmid DNA can also be complexed with proteins that target the plasmid DNA to specific tissues in the same way as certain proteins are taken up (endocytosed) by specific cells. Another plasmid-based technique involves 'shooting' the plasmid DNA on small gold beads into the cell using a 'gun'. Finally, muscle cells have the unusual ability to take up and express plasmid DNA.

Gene therapy approaches can be classified into direct and indirect methods. Direct methods using several of the above gene-transfer methods involve targeting tissues such as muscle, liver, lung or brain, as well as tumours, within the body. Indirect methods imply genetic manipulation of cells that have been removed from the body followed by transplantation of the genetically modified cells back into the body. Indirect approaches in conjunction with retroviral vectors are being developed to transfer genes into bone marrow cells, lymphocytes, hepatocytes, myoblasts and skin cells. Regardless of the approach, in order to judge the possible applications of a given gene delivery method, it is necessary to assess the total amount of gene product that can be produced, the efficiency and stability of gene expression, and the repeatability and safety of the procedure.

We have previously reported that pure RNA and DNA can be taken up by skeletal muscle cells *in vivo* without any special delivery mechanism[2]. By an incision on the anterior thigh, the quadriceps muscle in Balb/c mice was exposed and injected with either 100 μg pRSVCAT DMNA plasmid[3] or 100 μg bgCATbgA$_n$ RNA[4,5]. The RNA consists of the chloramphenicol acetyl transferase (CAT) coding sequences flanked by globin 5′ and 3′ untranslated sequences and a 3′ polyadenylate tract. The levels of expression were comparable to the levels achieved in fibroblasts transiently transfected with these vectors under optimal conditions using lipofectin.

The site of gene expression has been determined for the pRSVlacZ vector expressing the *Escherichia coli* β-galactosidase gene[6]. Seven days after a single injection of 100 μg pRSVlacZ DNA into mouse quadricep muscle, approximately 60 out of the 4000 muscle fibres comprising the muscle showed histochemical evidence of β-galactosidase activity. Longitudinal sections revealed continuous β-galactosidase staining within muscle cells for at least 400 μm. Positive staining within some individual muscle fibres was at least 1.2 mm deep on serial cross-sections.

To quantify the efficacy of gene expression more accurately, mouse quadricep muscles were injected with various amounts of βgLucβgA$_n$ RNA or pRSVL DNA constructs containing the firefly luciferase gene. A dose–response effect was observed. On the basis of the amount of DNA delivered, the efficiency of expression

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from the DNA vectors was similar in both transfected fibroblasts and injected muscles.

The time course of expression has also been investigated. Luciferase activity was assayed at various times after 100 μg of βgLucβgA$_n$ RNA or pRSVL DNA were injected. After RNA injection, the average luciferase activity reached a maximum of 74 pg at 18 h and then decreased to 2 pg at 60 h. In transfected fibroblasts, the luciferase activity was maximal at 8 h. After DNA injection into muscle, substantial amounts of luciferase were present for at least 60 days. The luciferase protein and the *in vitro* RNA transcript appear to have a half-life of less than 24 h in muscle. Therefore, the persistance of luciferase activity for 60 days in muscle after pRSVL DNA injection is not likely to be due to the stability of the luciferase protein or the stability of the *in vitro* RNA transcript.

Southern blot analysis of muscle DNA indicated that foreign DNA was present in the muscle tissue for at least 30 days. In muscle DNA digested with *Bam*HI (which cuts pRSVL once), the presence of a 5.6 kb band that corresponds to linearized pRSVL suggests that DNA is present either in a circular extrachromosomal form or in large tandem repeats of the plasmid integrated into the chromosome. In muscle DNA digested with *Bgl*I (which does not cut pRSVL), the presence of a band smaller than 10 kb and of the same size as the open circular form of the plasmid pRSVL implies that DNA is present extrachromosomally in a circular form. Extrachromosomal DNA of muscle extracts was prepared by the method of Hirt[7], modified by Pauza and Galindo[8]. The appearance of pRSVL DNA in Hirt supernatants and bacteria rendered ampicillin-resistant after transformation with Hirt supernatants also suggests that DNA is present in an unintegrated form. Overexposure of blots did not reveal smears of hybridizing DNA that would represent plasmid DNA integrated at random sites. The sensitivity of the pRSVL DNA in muscle to *Dpn*I digestion and its resistance to *Mbo*I digestion suggest that the bacterial methylation pattern is maintained and the DNA has not replicated in the muscle cell[9].

After these initial studies, more extensive studies were performed to explore the stability of plasmid DNA and expression in muscle for longer times[10]. Given the small amount of plasmid DNA in the muscles, a small percentage of DNA could have integrated without being detected on Southern blots. Because of possible deleterious effects from integration, we employed more sensitive methods to detect integration of plasmid DNA into the muscle chromosome, which involved cloning of plasmid DNA extracted from injected muscle. We were also interested in the amount of plasmid DNA and its methylation pattern over longer periods of time, assessed using the more sensitive polymerase chain reaction (PCR) method.

Stability of expression was assessed after 100 μg of either undigested, covalently closed circular (CCC) pRSVL or pRSVL made linear by digestion with *Bam*HI was transfected into 3T3 cells or injected into mouse quadricep muscle. Expression from the linear plasmid was less efficient in both systems. Luciferase activity was stable for at least 4 months after injection of the linear plasmid. Injection of CCC pRSVL resulted in stable luciferase expression for more than two years, which is practically the lifetime of the mouse.

The pRSVCAT DNA plasmid was used to determine whether stable expression occurs with another reporter gene (CAT). Substantial levels of CAT activity were present in most of the muscles, indicating that stable expression can occur with another reporter gene.

The pCMVLux plasmid was used for stability studies because of differences from the pRSVL plasmid such as the π suppressor gene for bacterial selection and the immediate-early cytomegalovirus promoter[11]. Luciferase expression persisted for at least 180 days after injection of pCMVLux.

In order to detemine whether the rate of cell division affects the stability of expression from plasmid DNA, pRSVL was transfected into rat-1 fibroblasts. After transfection, the cells were split into one fraction that continued to divide as they were continually passed prior to confluency. The other cell fraction was significantly less mitotic since fibroblasts reached confluency and were treated with dexamethasone[12]. In both dividing and confluent cells, luciferase expression was maximal at 7 days after transfection. Expression was not stable in either group. The decrease in activity was 5% per day in the unpassed cells and 40% per day in the passed cells. These results indicate that stability of expression is affected by the mitotic rate of the cells.

Quantitative PCR was used to determine the amount of pRSVL DNA in injected muscles over time. Southern blot analysis was not sensitive enough to detect plasmid DNA after longer periods of time. We found muscles containing concentrations of pRSVL DNA above background one year after injection. At none of the timepoints after injection was there any correlation between the amount of luciferase and pRSVL DNA in the muscle. These results demonstrate that plasmid DNA can persist in muscle in the long term. We used the PCR reaction in order to determine whether the plasmids were replicated or repaired while in the muscle for a long period of time. Plasmid DNA from bacteria contains methylated adenosine within the GATC recognition sites for *Dpn*I, *Mbo*I and *Sau*IIIA. Methylation of adenosine causes the efficiency of *Mbo*I to decrease and that of *Dpn*I to increase. *Sau*IIIA cleaves efficiently at this site regardless of methylation. We used primers to amplify a segment of pRSVL DNA containing four GATC restriction sites after digestion with the above enzymes. In all four muscle samples examined after 19 months, pRSVL DNA was amplified after *Dpn*I digestion but not after *Mbo*I digestion. These results indicate that the bacterial methylation pattern of the injected pRSVL DNA did not change after it has been maintained in muscle for at least 19 months.

Electroporation was used to clone plasmid DNA in muscle to determine its integration state. Previous experiments with pure CCC plasmid DNA electroporated alone or in 1.0 μg of total muscle or fibroblast DNA have shown that genomic DNA does not affect electroporation efficiency. Total cellular DNA from fibroblasts stably transfected with pRSVL (40 pg pRSVL per μg genomic DNA) served as a control for plasmid integration. Total DNA from muscles injected up to one year earlier was efficient in transforming DH10B bacteria. Digestion of plasmids from ampicillin-resistant colonies with several different restriction enzymes indicated that these colonies contained the pRSVL plasmid. Less than two ampicillin-resistant colonies per mg genomic DNA were obtained with uninjected control muscle. The ability of cellular DNA from pRSVL-injected muscle to

transform bacteria with pRSVL suggested that the pRSVL was maintained extrachromosomally for at least one year.

Further studies using bacterial electroporation were performed to determine whether any of the injected plasmid DNA had integrated. Total cellular DNA from fibroblasts stably transfected with pSV2Neo served as a positive control for integration. When this cellular DNA was digested with *Bgl*II (which cleaves pSV2Neo once within the neomycin resistance gene), ligated and electroporated, ampicillin-resistant colonies were obtained. About half of the plasmids isolated back from the ampicillin-resistant colonies were unaltered in size and were most likely derived from tandem repeats (transfected plasmid DNA typically integrates in tandem repeats). The remaining plasmids were altered in size and were probably derived from pSV2Neo at the junction between plasmid and chromosomal DNA. We examined muscles injected with the plasmid pUC19, expressing the β-galactosidase gene, for the ability to transform bacteria after electroporation. Loss of β-galactosidase expression would indicate disruption of the *lacZ* coding region and a possible integration event. Electroporation of muscle DNA without prior digestion yielded $<5\%$ white colonies. The muscle DNA was then digested with either *Pst*I or *Bam*HI restriction enzymes which cut pUC19 once within the *lacZ* gene. After ligation and electroporation, approximately 5% of 1800 colonies were white. Plasmid DNA was prepared from 78 white colonies. Restriction enzyme analysis and sequencing indicated that some of the plasmids from the colonies contained an insert within the *Pst*I or *Bam*HI site of pUC19, which probably arose during ligation and did not represent an integration event. Other plasmids from the white colonies were contaminating plasmids that were present in the laboratory. None of the plasmids cloned from the injected muscle contained plasmids altered in size and therefore no integration events were detected.

Increased efficiency of expression would be better obtained by studying how other tissues can take up and express genes introduced by direct injection. We therefore studied the levels of expression in several tissues that were surgically exposed and injected under direct visualization with plasmids containing various promoters and reporter genes[13]. No significant levels of gene expression were detected in brain, liver, spleen, uterus, stomach, lung or kidney. Only rat hearts contained levels of expression comparable to levels in skeletal muscle or transiently transfected fibroblasts. After injection with pRSVlacZ, β-galactosidase activity was localized to the myocardial cells. These results indicate that cardiac muscle, similarly to skeletal muscle, is able to take up and express plasmid DNA. However, expression was stable for only two weeks, as tested with three different reporter genes (luciferase, β-galactosidase and CAT) and with three different promoters (RSV, CMV, PGK). The difference in the stability of transfected DNA in skeletal and cardiac muscle tissues may reflect differences in their inherent cellular properties, such as the multinucleated nature of skeletal muscle fibres. The cellular environment that permits skeletal fibres to maintain multiple nuclei may also enable foreign DNA to persist. Stable expression of luciferase in rats under cyclosporin A treatment or with hereditary absence of the thymus suggests the involvement of immunological factors. The disappearance of both luciferase and pRSVL DNA after 2–3 weeks is consistent with destruction of the transfected cells as a result of an immune response.

We also examined the stability of luciferase expression in mdx mouse muscle[14]. This is the mouse model of Duchenne muscular dystrophy, characterized by ongoing degeneration of muscle fibres. The biochemical basis of the disease is the absence of the dystrophin protein from muscle fibres as a result of a mutation in the dystrophin gene on the X chromosome. Luciferase expression is not stable in mdx muscle, possibly due to loss of fibres by ongoing degeneration.

After injection of plasmid DNA containing the dystrophin gene, about 1% of muscle cells expressed the dystrophin protein[11]. This expression was stable for at least 6 months[10]. We have also shown that luciferase expression from plasmid DNA containing the dystrophin gene along with the luciferase gene is stable in mdx muscle[10]. This suggests that dystrophin expression improves myofibre survival in mdx mice.

It is clear that the efficiency of gene transfer has to be increased if it is to be applied to the treatment of genetic muscle diseases. However, even at the relatively low efficiency of expression that we are able to achieve at present, plasmid DNA transfer seems to be a very promising way of programming cells *in vivo* to secrete proteins for immunization purposes, or to produce proteins with enzymatic activity to clear toxic metabolites from the circulation.

It seems that the unique ability of muscle cells to take up DNA is not only due to the postmitotic state but also to other intrinsic characteristics, since other postmitotic cells do not show the same quality. It is unlikely that entry of the plasmid DNA is merely the result of muscle fibre injury, because β-galactosidase-expressing cells are seen far away from the injection site. Substantial expression of plasmid DNA is also seen in myotubes in culture which argues against the hypothesis of plasmid entry via gross membrane damage. The uptake process appear to be intrinsic to muscle. There is some experimental evidence that T tubules and caveolae – structures unique to muscle – may play an important role in DNA uptake. Electron microscopic images of muscle injected with gold-coated DNA show the gold preferentially localized to the T tubules and the caveolae as opposed to other cationic gold conjugates (PEG–gold, polylysine–gold) where this characteristic distribution cannot be seen. It is also worth mentioning that such tubules are common features of cardiac and skeletal muscle, both of which are capable of taking up and expressing plasmid DNA. There is no T-tubular system in smooth muscle and we have not found significant gene expression in organs rich in smooth muscle such as the stomach and uterus.

In order to try to increase gene expression, we studied the effects of pretreatment of the muscle with different agents before plasmid injection[13]. We found up to 40-fold increases in expression after pretreatment with bupivacaine. Bupivacaine (Marcaine) is a local amide anaesthetic that selectively destroys myofibres, while leaving satellite cells, the basement membrane and the vessels intact. Myonecrosis and phagocytosis of the necrotic material occurs, followed by vigorous regeneration. The muscle completely recovers and is undistinguishable from normal muscle after two weeks, except for central localization of nuclei. We found that

the optimal time for bupivacaine pretreatment is around 7 days before plasmid injection, and the concentration of the drug giving the best results is 0.5–0.75%. The high level of reporter gene expression persisted for a month, but decreased to that of non-pretreated muscle by two months after intramuscular plasmid injection. Temporary enhancement of foreign gene expression may be particularly advantageous in vaccination protocols employing intramuscular plasmid injection.

In summary, the direct injection of naked plasmid DNA without a special vehicle can permanently generate substantial levels of foreign genes in skeletal muscle. Although increased levels of expression are necessary for other applications, the levels achieved to date may be sufficient for immunization applications.

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Expression of Deletion-Containing Dystrophins in *mdx* Muscle: Implications for Gene Therapy and Dystrophin Function

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ABSTRACT

The expression of full-length dystrophin and various dystrophin deletion mutants was monitored in *mdx* mouse muscle after intramuscular injection of dystrophin-encoding plasmid DNAs. Recombinant dystrophin proteins, including those lacking either the amino terminus, carboxyl terminus, or most of the central rod domain, showed localization to the plasma membrane. This suggests that there are multiple attachment sites for dystrophin to the plasma membrane. Only those constructs containing the carboxyl terminus were able to stabilize dystrophin-associated proteins (DAP) at the membrane, consistent with other studies that suggest that this domain is critical to DAP binding. Co-localization with DAP was not necessary for membrane localization of the various dystrophin molecules. However, stabilization and co-localization of the DAP did seem to be a prerequisite for expression and/or stabilization of mutant dystrophins beyond 1 wk and these same criteria seemed important for mitigating the histopathological consequences of dystrophin deficiency. (***Pediatr Res* 37: 693–700, 1995**)

Abbreviations

DMD, Duchenne muscular dystrophy
PCR, polymerase chain reaction
pRSVDy, plasmid DNA encoding full length dystrophin
pRSVDy-B, plasmid DNA encoding Becker-like dystrophin
pSV40L, plasmid DNA encoding firefly luciferase
pRSVDy-SV40L, plasmid DNA encoding both full-length dystrophin plus luciferase
pRSVDy-B-SV40L, plasmid DNA encoding both Becker-like dystrophin plus luciferase
pRSVDy-A, plasmid DNA encoding amino-terminal domain-deleted dystrophin
pRSVDy-C, plasmid DNA encoding carboxyl-terminal domain-deleted dystrophin
pRSVDy-D, plasmid DNA encoding amino- and carboxyl-terminal domain-deleted dystrophin

Aberrations in dystrophin expression are responsible for DMD and Becker muscular dystrophy (1). It also appears that deficiencies of dystrophin-associated proteins may play an important role in DMD and other severe childhood myopathies (2). A promising cure for DMD is placement of the normal dystrophin cDNA into affected tissue. Previous transgenic studies in *mdx* mice—an animal model of DMD (3)—suggest that expression of recombinant dystrophin restores normal muscle morphology and function (4–6). Restoration of dystrophin expression in *mdx* muscle has also been observed after the injection of retroviral vectors (7), naked plasmid DNA (8), or adenoviral vectors (9). Dystrophin expression has been shown to improve myofiber survival in *mdx* muscle after dystrophin gene transfer either by injection of naked plasmid DNA (10) or adenoviral vectors (11).

The main issues concerning DMD gene therapy approaches are: *1*) transfer and expression of dystrophin in sufficient numbers of muscle fibers and *2*) prevention of disease progression by recombinant dystrophin expression. The transgenic *mdx* mouse studies imply that recombinant dystrophin expression can correct dystrophic muscle to the extent that it has normal muscle morphology and function (4–6). However, the results obtained with embryonic gene transfer may not extend to somatic gene transfer which would involve gene transfer at later development times. Somatic gene transfer using adenoviral vectors has resulted in stable dystrophin expression only when introduced into neonatal mice (12); a treatment situation difficult to mimic in all DMD patients. Also, limitations on the

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size of the gene to be transferred is currently a problem with adenoviral vectors and has resulted in their use to transfer only a Becker-like dystrophin to dystrophic muscle (9,11). Using adenoviral vectors, it is at present not possible to compare the efficacy between Becker-like and full-length dystrophin expression in dystrophic muscle. Postnatal gene transfer into muscle by intramuscular injection of plasmid DNA has been used to transfer and express genes encoding both the Becker-like and full-length dystrophins in dystrophic muscle (8,10). Unfortunately, it remains difficult to assess the therapeutic value of this treatment on the muscle as a whole since less than 1% of the myofibers express dystrophin after intramuscular injection. But this approach does provide sufficient dystrophin expression to assess its therapeutic value at the myofiber level (10).

To further characterize the functional relationships of the various domains of dystrophin, and the importance of these domains in relationship to DMD gene therapy, we examined the expression of various deletion-containing dystrophins in *mdx* muscle after the intramuscular injection of plasmid DNA. The effect of dystrophin expression on dystrophin-associated protein expression, myofiber survivability, and percentage of centrally located nuclei were used to assess the functionality of the deleted dystrophin domains with regard to dystrophin function or therapeutic value toward DMD gene therapy. Previous studies have shown that the stability of expression of dystrophin in combination with a reporter gene can be used as an indirect indication of myofiber survival (10,11).

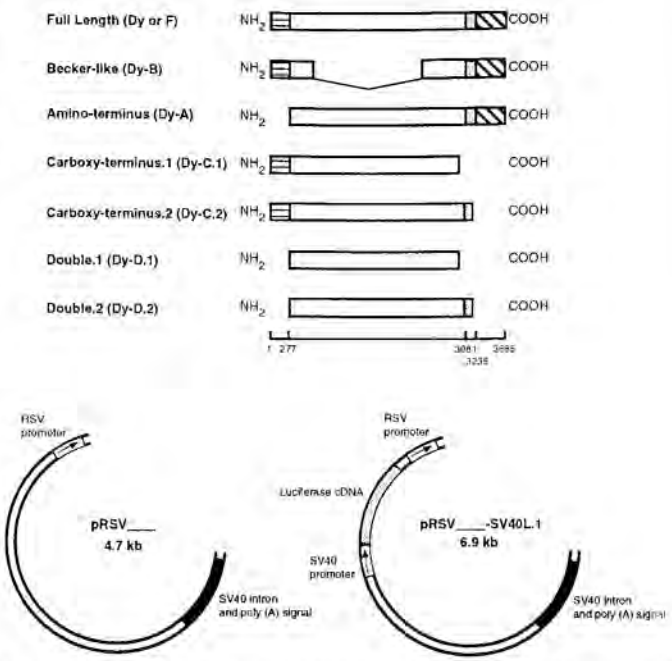

Figure 1. Diagrammatic representation of the dystrophin molecules encoded by the various expression plasmids injected into *mdx* muscle. The full-length dystrophin sequence and the borders of its varying domains was based on previously published sequence (36). The approximate amino acid boundaries corresponding to the amino-terminal (actin-binding, *horizontal shading*), spectrin-like repeats (*white*), cysteine-rich (*stippled shading*), and carboxyl-terminal (*diagonal shading*) domains are noted below the diagram of the double.2 molecule. The cDNA encoding each of these dystrophin molecules was ligated into either the pRSV or the pRSV-SV40L.1 vectors previously described (10).

METHODS

Mouse strains. The C57BL/6, Balb/c, and ICR strains were obtained from Harlan Sprague-Dawley (Indianapolis, IN). The mdx^{4cv} and mdx^{5cv} strains (gift of V. Chapman) having indistinguishable phenotypes (13,14) were used interchangeably and are simply referred to as *mdx* mice (10) unless otherwise indicated. These specific strains were used because they have substantially lower number of revertants than the original *mdx* strain (14).

Plasmid construction, preparation, and injection. Plasmid DNAs pRSVDy, pRSVDy-B, pSV40L.1, pSV40L.2, and pRSVDy-SV40L.1 or pRSVDy-B-SV40L.1, were identical to those previously described (10). The pRSVDy-B construct encodes a dystrophin molecule lacking amino acids 664–2366 (Fig. 1, Becker-like). The pUC19 plasmid was obtained commercially (Life Technologies, Inc., Bethesda, MD).

An expression construct, pRSVDy-A, encoding a dystrophin lacking amino acids 1–277 (Fig. 1, amino terminus) was prepared using PCR primers Dys-*Not*I (5′-GGGCCCGCGGCCGCAATGATCACGGTCAGTCTAGCA-3′) and Dys-*Aat*II (5′-CCCGGGCTGACGTCCAGTCTTTATC-3′) to amplify the dystrophin cDNA region encoding amino acids 277–1526 from pRSVDy. The Dys-*Not*I primer has an ATG site located within it so that encoded dystrophin will start with a methionine and then continue with residue 277. This ensures that this mutant has identical 5′ sequences as the other mutant constructs not containing 5′ deletions. The resulting 3745-bp product was ligated into pCRII (Invitrogen) and the resulting plasmid digested with NotI and AatII. This fragment was ligated into pRSVDy (previously digested with *Not*I and *Aat*II) to yield pRSVDy-A.

Two carboxyl-terminal dystrophin mutant constructs, pRSVDy-C.1 and pRSVDy-C.2, were also prepared. Construct pRSVDy-C.1 was prepared by digesting pRSVDy with *Xho*I and *Sal*I, and then ligating the ends together. The pRSVDy-C.1 construct encodes a dystrophin molecule lacking amino acids 2975–3685 (Fig. 1, carboxyl terminus.1). Construct pRSVDy-C.2 was prepared by first amplifying a DNA fragment encoding dystrophin amino acids 2829–3195 by PCR using pRSVDy as template and Dys-*Ngo*MI (5′-CCCGGGGCCGGCAGGCACCTATTGG-3′) and Dys-*Sal*I (5′-GGGCCCGTCGACC TACTAGACACGGATCCTCCCTGTTCG-3′) primers. This PCR fragment was ligated into pCRII and the resulting plasmid digested with *Ngo*MI and *Sal*I. The digested fragment was then cloned into pRSVDy previously digested with *Ngo*MI and *Sal*I to yield pRSVDy-C.2. The pRSVDy-C.2 construct encodes a dystrophin molecule lacking amino acids 3196–3685 (Fig. 1, carboxyl terminus.2).

Constructs encoding dystrophin lacking both the amino- and carboxyl-terminal regions were prepared by combining the schemes described above. Construct pRSVDy-D.1 encodes a dystrophin molecule lacking both the amino- and carboxyl-terminal domains described for both pRSVDy-A and pRSVDy-C.1 (Fig. 1, double.1). It was prepared by digesting pRSVDy-A with *Xho*I and *Sal*I and ligating the resulting ends together.

Construct pRSVDy-D.2 encodes a dystrophin molecule lacking the regions defined by both pRSVDy-A and pRSVDy-C.2 (Fig. 1, double.2). It was constructed by inserting the *Ngo*MI/*Sal*I fragment of pRSVDy-C.2 into pRSVDy-A.

The cDNA sequences encoding these various dystrophin molecules were placed in expression vectors which either lacked or contained a region capable of encoding luciferase (Fig. 1). Vectors containing both luciferase and dystrophin cDNA have the SV40L.1 notation added to the plasmid (*i.e.* the plasmid encoding both the full-length dystrophin and luciferase was termed pRSVDy-SV40L.1). These vectors serve as indicators of myofiber survival since luciferase expression persists in *mdx* muscle only after transfer and expression of either full-length or Becker-like dystrophin (10).

All plasmids were purified by alkaline lysis and two CsCl gradients as previously described (15). Plasmid DNA injections into mouse quadriceps were done in 100 μL of normal saline (10). Specific amounts of plasmid DNA injected per quadriceps are given in the figure legends.

Immunohistochemistry, luciferase, and β-galactosidase assays. Dystrophin immunohistochemistry was performed using antibodies which recognized either the rod (16) (generously provided by L. Kunkel), amino-terminal or carboxyl-terminal domains of dystrophin (17) in combination with biotin-conjugated sheep anti-rabbit secondary antibody (Amersham Corp., Arlington Heights, IL) and streptavidin-Texas Red (Life Technologies). The number of dystrophin-positive myofibers per muscle was determined as previously described (8,10). Antibodies specific for α-dystroglycan (156-kD dystrophin-associated glycoprotein) (18), β-dystroglycan (43-kD dystrophin-associated glycoprotein) (18,19), and adhalin (50-kD dystrophin-associated glycoprotein) (20) were also previously described.

The proportion of dystrophin-positive fibers containing centrally located nuclei was determined as previously described (8). Briefly, *mdx* muscle sections were processed for dystrophin staining (as previously described) and co-stained with propidium iodide (Sigma, St. Louis, MO) to visualize both dystrophin-positive fibers and nuclei (21).

Determinations of luciferase and β-galactosidase expression were done using previously described methods (22,23).

The *t* test was used for statistical analysis.

RESULTS

Full-length and Becker-like dystrophins. Immunohistochemical staining of normal skeletal muscle with appropriate antibodies reveals dystrophin and dystrophin-associated proteins as a continuous staining along the plasmalemma of every muscle fiber. Except for rare revertant fibers, this staining can not be seen in *mdx* muscle.

The number of dystrophin-positive myofibers was determined at 1 wk and 2 mo after the intramuscular injection of 400 μg of pRSVDy and pRSVDy-B into *mdx* mouse muscle. Control muscles were injected with 400 μg of the plasmid pUC19 under similar conditions. The number of dystrophin-positive myofibers was corrected for revertant fibers by subtracting the number of dystrophin-positive fibers observed in muscles injected with the control plasmid pUC19 which averaged 4 ± 1 and 17 ± 0.5 (mean ± SE) at 1 wk and 2 mo after injection, respectively.

Expression of the full-length and Becker-like dystrophin persisted for at least 2 mo after intramuscular injection of the corresponding expression plasmid (Fig. 2). Dystrophin was visualized by immunohistochemical staining with dystrophin antibodies specific for either the amino-terminal, rod or carboxyl-terminal domain (Fig. 3*a–c*). Full-length dystrophin localized to the sarcolemmal membrane (Fig. 3*a–c*) and the localization of both the full-length and Becker-like dystrophin was similar (data not shown). The number of dystrophin-positive fibers increased slightly from 1 wk to 2 mo after plasmid DNA injection for samples expressing either the full-length or Becker-like dystrophins (Fig. 2). Concomitant with full-length dystrophin expression was the co-localization of the dystrophin-associated proteins adhalin (Fig. 3*d*), α-dystroglycan and β-dystroglycan (Fig. 4*b* and *d*). Similar observations were made in *mdx* muscle expressing Becker-like dystrophin (data not shown).

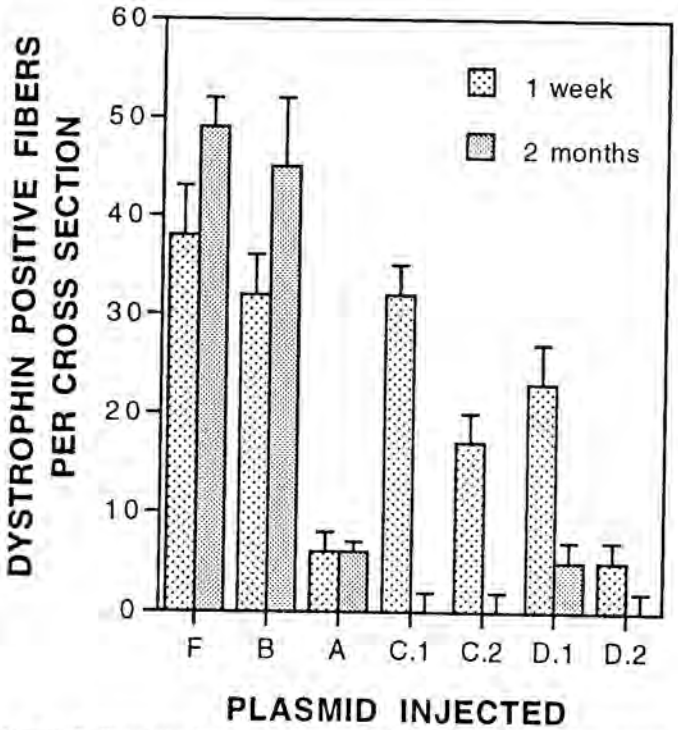

Figure 2. Duration of expression for the various dystrophin molecules in *mdx* muscle. Mice, 4–6 wks of age, were given intramuscular injections of 400 μg of full-length dystrophin expression plasmid pRSVDy (*F*), 400 μg of Becker-like expression plasmid pRSVDy-B (*B*), 400 μg of amino-terminally deleted dystrophin expression plasmid pRSVDy-A (*A*), 400 μg of carboxyl-terminally deleted dystrophin expression plasmid pRSVDy-C.1 (*C.1*), 400 μg of carboxyl-terminally deleted dystrophin expression plasmid pRSVDy-C.2 (*C.2*), 400 μg of amino- and carboxyl-terminally deleted dystrophin expression plasmid pRSVDy-D.1 (*D.1*), or 400 μg of amino- and carboxyl-terminally deleted dystrophin expression plasmid pRSVDy-D.2 (*D.2*). Mice were assayed for dystrophin expression at 1 wk and 2 mo after plasmid injection. At least six different quadriceps muscles (from at least six mice) were examined for each treatment group. Values represent the mean number of dystrophin-positive fibers observed per muscle at the site of plasmid DNA injection, corrected for the number of revertant fibers. *Bars* indicate SE.

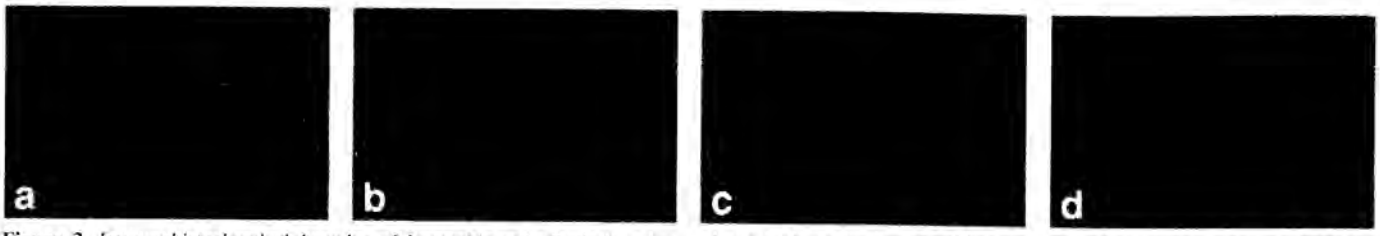

Figure 3. Immunohistochemical detection of dystrophin and adhalin in *mdx* muscle 1 wk after injection of full-length dystrophin expression plasmid pRSVDy. Serial sections stained with antibodies specific for the amino-terminal (*a*), spectrin-like repeat (*b*), and carboxyl-terminal (*c*) domains of dystrophin and for the dystrophin-associated protein adhalin (*d*). Magnification, ×62.5.

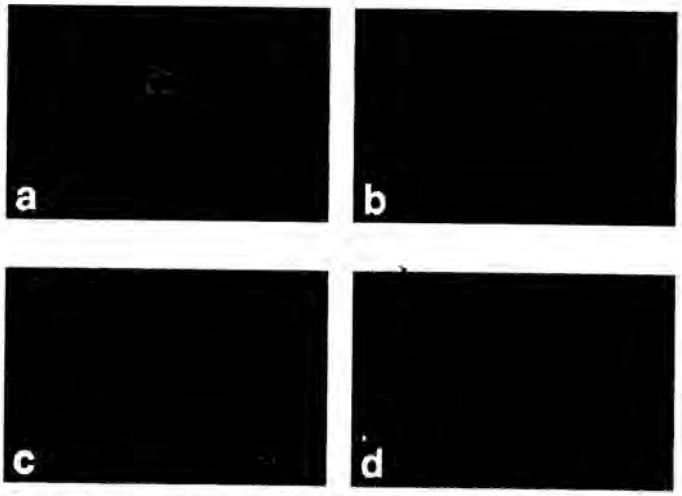

Figure 4. Immunohistochemical colocalization of dystrophin with dystrophin-associated proteins α- and β-dystroglycan in *mdx* muscle 1 wk after injection of full-length dystrophin expression plasmid pRSVDy. Two pairs of serial sections (*a*, *b*, and *c*, *d*) are shown. Sections (*a*) and (*c*) are stained with antibody specific for the spectrin-like repeat (rod), domain of dystrophin. Sections (*b*) and (*d*) were stained with antibodies specific for α- or β-dystroglycan, respectively. Magnifications are ×62.5 for (*a*, *b*) and ×40 for (*b*, *d*).

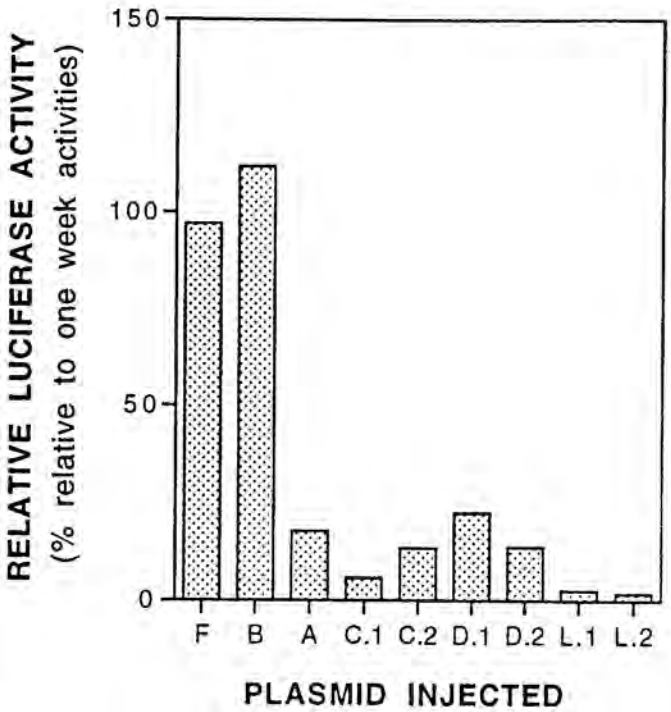

Figure 5. Stability of luciferase expression in *mdx* mouse muscle. Mice, 4–6 wk of age, were given intramuscular injections of 50 μg of pRSVDy-SV40L.1 (*F*), 50 μg of pRSVDy-B-SV40L.1 (*B*), 50 μg of pRSVDy-A-SV40L.1 (*A*), 50 μg of pRSVDy-C.1-SV40L.1 (*C.1*), 50 μg of pRSVDy-C.2-SV40L.1 (*C.2*), 50 μg of pRSVDy-D.1-SV40L.1 (*D.1*), 50 μg of pRSVDy-D.2-SV40L.1 (*D.2*), 10 μg of pSV40L.1 (*L.1*), or 10 μg of pSV40L.2 (*L.2*). Mice were assayed for luciferase expression at 1 wk and 2 mo after plasmid injection. At least six different quadriceps muscles (from at least six mice) were examined for each treatment group. Values represent the relative luciferase activity remaining at 2 mo compared with levels observed at 1 wk for each sample.

The levels of luciferase activity were determined at 1 wk and 2 mo after injection of the plasmid containing both the dystrophin and luciferase genes. Luciferase expression persisted at similar levels between 1 wk and 2 mo after plasmid DNA injection in *mdx* muscle expressing either full-length or Becker-like dystrophin (Fig. 5). Another indication of improved *mdx* myofiber health was the significantly decreased number of centrally located nuclei observed in *mdx* muscle expressing either full-length or Becker-like dystrophin 2 mo after plasmid DNA injection (Fig. 6).

Amino terminus-deleted dystrophin. The number of myofibers expressing a dystrophin which lacked its actin-binding domain was very low at both 1 wk and 2 mo in *mdx* muscle after intramuscular injection of the respective plasmid (Fig. 2). Using a panel of antibodies specific for different domains of dystrophin, revertant fibers were distinguished from those expressing the amino-terminal domain-deleted dystrophin. Those fibers expressing the amino-terminal domain-deleted dystrophin were visualized with dystrophin antibodies specific for the carboxyl-terminal domain (Fig. 7*b*), but were not visualized with the amino-terminal domain specific antibody (Fig. 7*a*). This dystrophin molecule localized to the sarcolemmal membrane and co-localized with the dystrophin-associated protein α-dystroglycan (Fig. 7*c*). Co-localization with adhalin was also observed during the expression of the amino-terminal domain-deleted dystrophin (data not shown). Luciferase expression in *mdx* muscle expressing the amino-terminal domain-deleted dystrophin did not persist to the same degree as that observed in *mdx* muscle expressing either the full-length or Becker-like dystrophin (Fig. 5). The percentage of dystrophin-positive fibers containing centrally located nuclei was greater in *mdx* muscle expressing the amino-terminal domain-deleted dystrophin number than *mdx* muscle expressing either the full-length or Becker-like dystrophin. But, this percentage was less than that observed in *mdx* muscle injected with control plasmid, pUC19 (Fig. 6).

Carboxyl terminus-deleted dystrophins. Expression of dystrophins containing carboxyl-terminal domain deletions was observed at 1 wk after intramuscular injection of the corresponding plasmid DNA but did not persist at 2 mo after

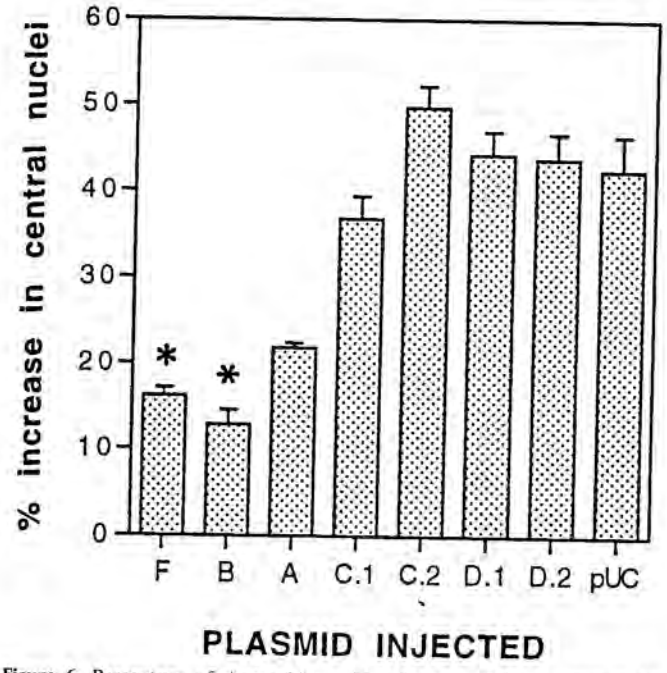

Figure 6. Percentage of dystrophin-positive fibers containing centrally located nuclei 2 mo after intramuscular injection of dystrophin-encoding plasmid DNA into *mdx* muscle. Mice, 4–6 wk of age, were given intramuscular injections of 400 μg of full-length dystrophin expression plasmid pRSVDy (*F*), 400 μg of Becker-like expression plasmid pRSVDy-B (*B*), 400 μg of amino-terminally deleted dystrophin expression plasmid pRSVDy-A (*A*), 400 μg of carboxy-terminally deleted dystrophin expression plasmid pRSVDy-C.1 (*C.1*), 400 μg of carboxyl-terminally deleted dystrophin expression plasmid pRSVDy-C.2 (*C.2*), 400 μg of amino- and carboxyl-terminally deleted dystrophin expression plasmid pRSVDy-D.1 (*D.1*), 400 μg of amino- and carboxyl-terminally deleted dystrophin expression plasmid pRSVDy-D.2 (*D.2*), or 400 μg of pUC19 (*pUC*). At least 250 dystrophin-positive fibers observed from at least four different muscles (from at least four mice) were examined for each sample. (*Statistically significant at $p < 0.05$.)

injection (Figs. 2 and 8). Using a panel of antibodies specific for either the amino-terminal, rod or carboxyl-terminal domains of dystrophin, it was possible to distinguish revertant fibers from those expressing the carboxyl-terminally deleted dystrophins. Fibers expressing the carboxyl-terminal domain-deleted dystrophin were visualized with the anti-dystrophin antibodies specific for the amino-terminal (Fig. 8*a*) and rod (Fig. 8*b*) domains, but not with the antibodies specific for the carboxyl-terminal domain (Fig. 8*c*). These truncated dystrophins localized to the sarcolemmal membrane but α-dystroglycan sarcolemmal expression was not restored by expression of this construct (Fig. 8*d*). Sarcolemmal expression of the other dystrophin-associated proteins studied was also not restored (data not shown). Fewer myofibers expressed dystrophin lacking a portion of the cysteine-rich domain and its carboxyl-terminal domain (Fig. 1, C.1) compared with myofibers expressing either the full-length, Becker-like, or dystrophin with the entire cysteine-rich domain and its carboxyl-terminal domain deleted (Fig. 1, C.2) at 1 wk after plasmid DNA injection (Fig. 2). Luciferase expression did not persist to the same degree in *mdx* muscle expressing the carboxyl-terminal domain-deleted dystrophin compared with that expressing either the full-length, Becker-like, or actin-binding domain-deleted dystrophins (Fig. 5). Compared to *mdx* muscle injected with pUC19, muscle injected with the carboxyl-terminally deleted dystrophin constructs had little difference in the number of dystrophin-positive fibers containing centrally located nuclei (Fig. 6).

Amino and carboxyl-terminus-deleted dystrophins. Expression of dystrophins lacking both the amino-terminal domain and the carboxyl-terminal domain was observed only at 1 wk after plasmid DNA injection into *mdx* muscle (Figs. 2 and 9). At this time, these double-deleted dystrophins localized to the sarcolemma and could be differentiated from revertant fibers on the basis of their reactivity with only the antibody specific for the rod domain of dystrophin (Fig. 9*b*). No dystrophin-associated proteins were observed in *mdx* muscle expressing these molecules (Fig. 9*d* and data not shown). Also, *mdx* muscle expressing the double-deleted dystrophins did not show persistent luciferase expression (Fig. 5) or decrease in the number of centrally located nuclei observed 2 mo after plasmid DNA injection (Fig. 6).

DISCUSSION

All the dystrophin constructs studied were expressed at 1 wk after plasmid DNA injection in *mdx* muscle to different extents (Figs. 2–4 and 7–9). The low level of revertant fibers in the particular strain of *mdx* mice used in this study (13) and the observation that revertant fibers do not show cytoplasmic

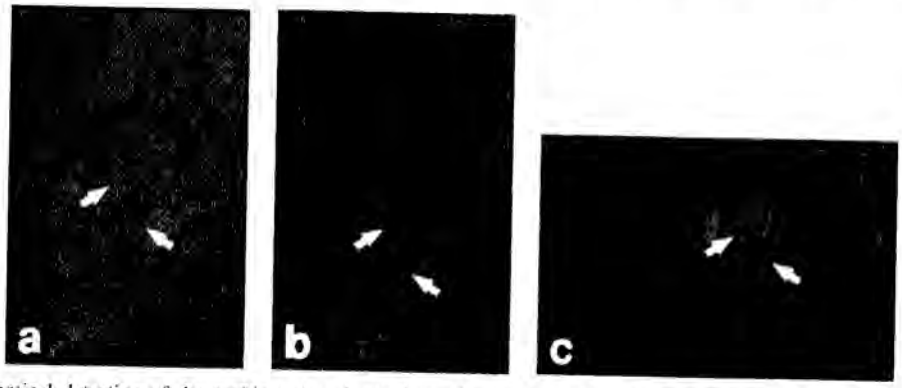

Figure 7. Immunohistochemical detection of dystrophin and α-dystroglycan in *mdx* muscle 1 wk after injection of amino-terminally deleted dystrophin expression plasmid pRSVDy-A. Serial sections stained with antibodies specific for the amino-terminal (*a*), or carboxyl-terminal (*b*) domains of dystrophin and antibody specific for α-dystroglycan (*c*). *Arrows* indicate the presumably transfected fibers expressing amino-terminally deleted dystrophin molecules. Magnification was ×40 except for (*a*) which was ×62.5.

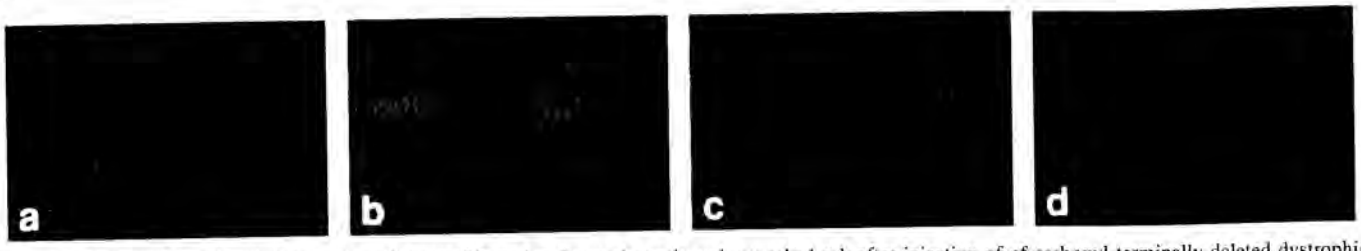

Figure 8. Immunohistochemical detection of dystrophin and α-dystroglycan in *mdx* muscle 1 wk after injection of of carboxyl-terminally deleted dystrophin expression plasmid pRSVDy-C.1. Serial sections stained with antibodies specific for the amino-terminal (*a*), spectrin-like repeat (*b*), and carboxyl-terminal (*c*) domains of dystrophin, and for for α-dystroglycan (*d*). Magnification, ×62.5.

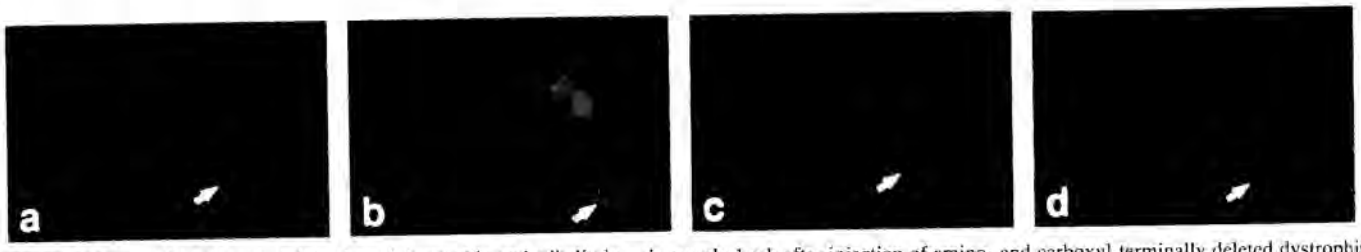

Figure 9. Immunohistochemical detection of dystrophin and adhalin in *mdx* muscle 1 wk after injection of amino- and carboxyl-terminally deleted dystrophin expression plasmid pRSVDy-D.1. Serial sections stained with antibodies specific for the amino-terminal (*a*), spectrin-like repeat (*b*), and carboxyl-terminal (*c*) domains of dystrophin and for adhalin (*d*). *Arrows* indicate a presumed revertant fiber. Magnification, ×40.

dystrophin staining (8) suggested that full-length and Becker-like dystrophin expression resulted from the cDNA within the injected plasmid DNA. Using a panel of antibodies specific for either the amino-terminal, rod, or carboxyl-terminal domains of dystrophin, it was possible to distinguish revertant fibers from those expressing either the amino- or carboxyl-terminally deleted dystrophins (Figs. 6–9). The differentiation of myofibers expressing the deletion-containing dystrophins from revertants further suggested that dystrophin expression and gene transfer did occur in *mdx* muscle after intramuscular plasmid DNA injection.

As previously reported (8–11), both the full-length and Becker-like dystrophins were localized at the sarcolemmal membrane (Fig. 3–4). These results were consistent with the sarcolemmal localization of dystrophin in Becker dystrophy patients that express a dystrophin lacking regions of the rod domain (34,35). After injection of the dystrophin genes lacking the amino or carboxyl terminus, the respective dystrophin proteins also were localized to the sarcolemmal membrane (Fig. 7–8). These results were consistent with observations in DMD patients in which dystrophins lacking either the amino- or carboxy terminus were localized to the sarcolemmal membrane (24–33). Similarly, independent localization of dystrophin amino and carboxyl terminus to the cell membrane of *mdx* muscle cells has recently been reported (45). Both our results and the human studies suggest that neither the amino-terminal nor the carboxyl-terminal domains of dystrophin are completely necessary for trafficking dystrophin to the membrane. These results suggest that there may be multiple regions within the dystrophin molecule responsible for its trafficking to the membrane. It is not unlikely that the portion of the rod domain not deleted from the Becker-like dystrophin also contains sarcolemmal localization cues for dystrophin and may suggest the mechanism by which the double deleted molecules localized to the membrane.

Another important indication of dystrophin function after plasmid injection is its ability to restore expression of the dystrophin-associated proteins. Expression of either the full-length or Becker-like dystrophins resulted in restoration and co-localization with the dystrophin-associated proteins in individual myofibers. This is in agreement with the expression of dystrophin-associated proteins in transgenic mice expressing full-length dystrophin (4–6). Although low numbers of dystrophin-positive myofibers were observed after the injection of plasmids encoding the amino terminus-deleted dystrophin (Fig. 2), co-localization of dystrophin-associated proteins and amino terminus-deleted dystrophin were observed (Fig. 7). However, no dystrophin-associated proteins co-localized with the fibers expressing the carboxyl-terminally deleted dystrophins (Fig. 8). This suggests that the carboxyl-terminal domain of dystrophin was essential for restoration and co-localization with the dystrophin-associated proteins. These results are in agreement with the ability of the carboxyl-terminal domain of dystrophin to complex with dystrophin-associated proteins (37–41).

We have previously shown that stability of dystrophin or luciferase expression can be used as indirect indication of myofiber survival in *mdx* mouse muscle (10). Expression of the full-length and Becker-like dystrophin persisted for at least 2 mo after intramuscular plasmid DNA injection (Fig. 2). Also in agreement with our previous study (10), co-expression of luciferase with the full-length and Becker-like dystrophin enabled stable expression of luciferase. These results also agree with similar findings using adenovirus-mediated transfer of Becker-like dystrophin (9,11). In contrast, co-expression of luciferase with the amino or carboxy terminus-deleted dystrophins did not enable stable expression of luciferase (Fig. 5). These results are consistent with the Duchenne phenotype observed in patients expressing dystrophins lacking either the amino or carboxyl terminus (24–33).

It was of interest that expression of the amino terminus-deleted dystrophin was not able to improve myofiber survivability or decrease the number of centrally located nuclei even though it enabled expression of the dystrophin-associated proteins (Figs. 2 and 5–7). This suggests that expression of the dystrophin-associated proteins and their interaction with the carboxyl-terminal domain of dystrophin was not sufficient to correct for the dystrophic phenotype in *mdx* muscle expressing the amino-terminal domain-deleted dystrophin. However, only those dystrophin constructs that restored dystrophin-associated protein expression resulted in improved myofiber survivability and a decrease in central nuclei. Most likely, myofiber survival requires a network of protein interactions encompassing the amino terminus of dystrophin and cytoplasmic elements such as actin (42), the carboxyl terminus of dystrophin and the dystrophin-associated proteins (37–41), and the dystrophin-associated proteins and extracellular elements such as laminin (18,43,44).

In summary, of the dystrophin molecules studied, only the full-length or Becker-like dystrophins exhibited long-term expression, co-localization with dystrophin-associated proteins, and persistent luciferase expression after plasmid DNA injection into *mdx* muscle (Figs. 2–5 and 7–9). This suggests that those *mdx* myofibers expressing either of these dystrophins were less susceptible to myofiber degeneration. Another indication of improved myofiber health was the decreased number of centrally located nuclei observed in *mdx* muscle expressing either full-length or Becker-like dystrophin (Fig. 6). Restoration of normal muscle morphology and function has been previously observed in transgenic *mdx* mice expressing dystrophin (4–6). This study suggests that after intramuscular injection of plasmid DNA, *mdx* myofibers expressing either full-length or Becker-like dystrophin are corrected for dystrophin deficiency and other secondary effects associated with dystrophin deficiency. Thus postnatal gene transfer may be beneficial to DMD patients provided that sufficient numbers of the myofibers express either the full-length or Becker-like DMD gene.

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MELLÉKLET (II)

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Pharmacological enhancement of in vivo foreign gene expression in muscle

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Intramuscular injection of naked plasmid DNA provides a means for gene transfer and expression in striated muscle. In this study, the effects of treating muscle with normal saline, etidocaine, mepivacaine, acetic anhydride, sodium bicarbonate, Notechis scutatus venom, cardiotoxin and bupivacaine before plasmid DNA injection on foreign gene expression were evaluated. Dose dependence, strain and species specificity, the time interval between pharmacological agent and plasmid DNA injection, the stability of gene expression and the fate of the injected plasmid DNA were studied using reporter gene expression, by histological examination and semi-quantitative polymerase chain reaction. Of the various agents tested, the best enhancement of foreign gene expression occurred in muscle treated with 0.75% bupivacaine five to seven days before plasmid DNA injection. Rat and mouse quadriceps muscle treated with 0.75% bupivacaine had levels of luciferase activity four- to 40-times greater than non-bupivacaine-treated muscle. Also, β-galactosidase expressing myofibers were observed throughout the length of the muscle in samples treated with 0.75% bupivacaine before reporter gene injection. Muscle treated with 0.75% bupivacaine fully recovered from the degeneration caused by its injection with no long-term effects histologically. The heightened level of reporter gene expression persisted in 0.75% bupivacaine-treated muscle for one month, but decreased to that of non-bupivacaine-treated muscle by two months after plasmid DNA injection. Enhancement of foreign gene expression may be particularly advantageous in vaccination protocols employing intramuscular plasmid injection.

Introduction

Genes transferred into skeletal and cardiac muscle *in vivo* by intramuscular injection of naked plasmid DNA can be expressed for at least 19 months [1–8]. The plasmid remains in a non-replicative, unintegrated, circular form [1, 9]. Plasmid DNA may enter striated muscle fibers by an active process following its injection [10]. Transferring genes directly into muscle using plasmid DNA has advantages over techniques using viral vectors. Unlike viral vectors, plasmid DNA accommodates larger segments of DNA, cannot yield replication-competent virus and creates no immune complications if properly purified [7]. The main obstacle associated with the plasmid DNA injection technique has been obtaining clinically sufficient levels of gene expression [3].

It has been demonstrated that treating muscle with bupivacaine prior to retroviral injection improved gene transfer, presumably by increasing the population of mitotically active cells in the muscle [11]. Bupivacaine (Marcaine), an amide local anesthetic, selectively destroys myofibers immediately following its injection into skeletal muscle of mice, rats, monkeys and humans, but muscle satellite cells, vascular endothelial cells or extracellular matrix are spared [12–16]. Local anesthetics destroy myofibers by increasing intracellular calcium concentration following interaction with the ryanodine receptor or other ion channels [17–22]. Myonecrosis, and phagocytosis occur by two days after bupivacaine injection followed by satellite cell (muscle stem cell) proliferation and myotube formation 1–3 days later [13]. The muscle completely recovers from the bupivacaine-induced damage two weeks after its injection and single bupivacaine injections appear to result in no long-term muscle abnormalities or histology [23]. Thus bupivacaine, a local anesthetic extensively used to induce muscle regeneration, may provide a means to enhance foreign gene expression when used in conjunction with direct *in vivo* gene therapy approaches.

In this study, the effects of treating muscle with bupivacaine and other compounds prior to plasmid DNA injection on reporter gene expression were examined. Our observations suggest that treatment of muscle with 0.75% bupivacaine (7.5 mg/ml) before plasmid DNA injection increases the level of foreign gene expression up to 40-fold compared to levels in non-treated muscle.

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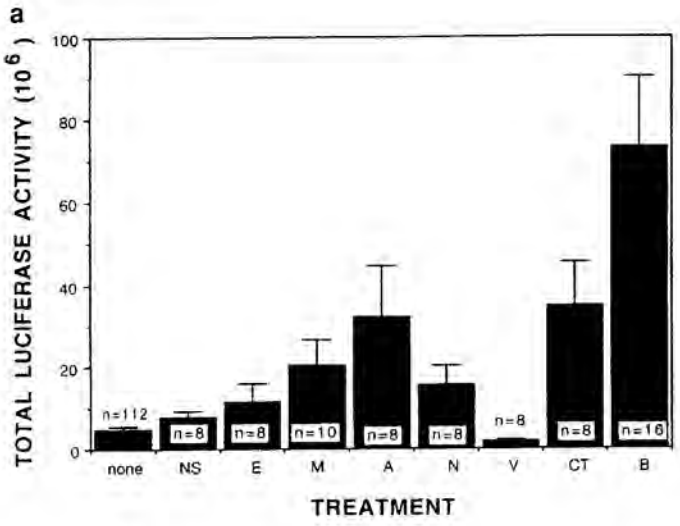
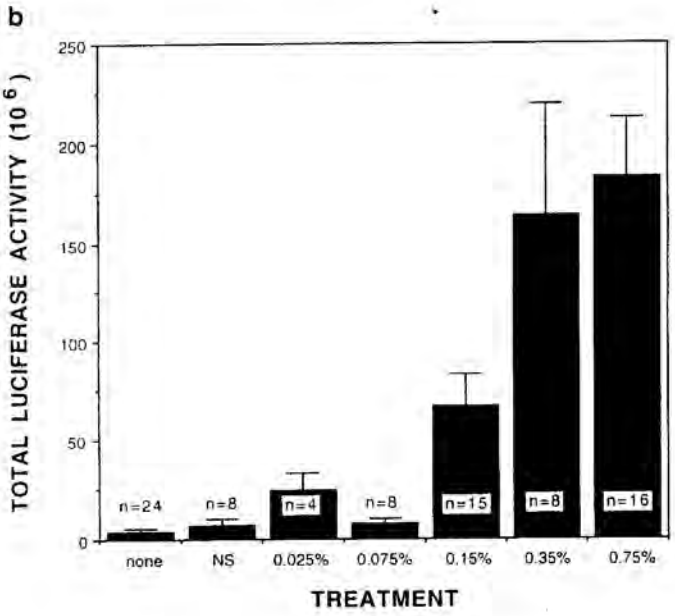
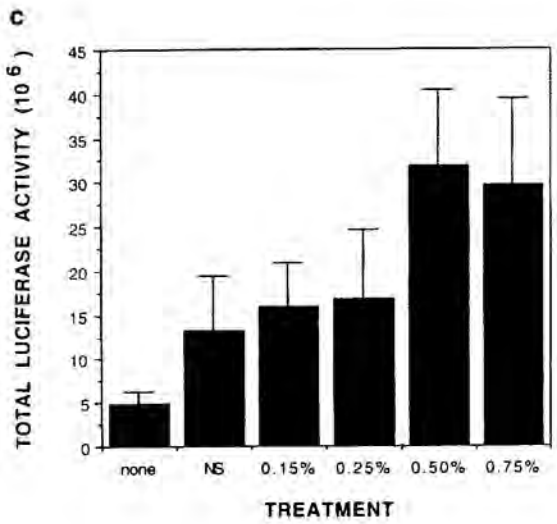
Results

Luciferase expression in mouse muscle treated with various agents

ICR mouse muscle injected with 10 μg pBS.RSVL 5 days after treatment with a variety of compounds was assayed for luciferase activity 2 weeks following plasmid DNA injection (Figure 1a). In addition, for each







of these treatments at least two muscles were examined at 2 and 14 days after the injection of these compounds to determine the extent of myonecrosis, phagocytic infiltration and muscle regeneration. Treatment with other local amide anesthetics, such as etidocaine and mepivacaine, increased expression only 1.5- and 2.7-fold, respectively, over muscle treated with normal saline (Figure 1a) even though the extent of myofiber damage, phagocytic infiltration and regeneration caused by each was similar to that of bupivacaine (data not shown). Compared to treatment with normal saline, treatment with 0. 1 M acetic anhydride pH 5.0 (A) or 0. 1 M sodium bicarbonate pH 9.0 (N) increased luciferase activity 4.2- and 2.0-fold, respectively. Both the acid and base treatments caused extensive myonecrosis and phagocytic infiltration followed by muscle regeneration similar to that previously reported [24]. The greatest enhancement of luciferase activity, besides that obtained with 0.75% bupivacaine, occurred in cardiotoxin-treated muscle (Figure 1a). Cardiotoxin also caused extensive myonecrosis and phagocytic infiltration followed by muscle regeneration as described previously [25]. Treatment with cardiotoxin increased luciferase activity 4.6-fold compared with that of normal-saline-treated muscle, whereas 0.75% bupivacaine treatment increased luciferase activity 9.6-fold (Figure 1a). Further optimization of cardiotoxin treatment could potentially increase luciferase activity levels to those obtained with 0.75% bupivacaine treatment. The lowest amount of luciferase activity occurred in muscle treated with *Notechis scutatus scutatus* venom (V) which may have resulted from a specific action of this toxin on the muscle [26].

Luciferase activity in mouse and rat muscles treated with bupivacaine

Balb/c mouse quadriceps were treated with various concentrations of bupivacaine 5 days before intramuscular injection of 10 μg pBS.RSVL plasmid DNA. Luciferase activity in muscle treated with 0.75% bupivacaine was 182×10^6 LU (light units)/ quadriceps, which was approximately 40-fold greater than the 4.24×10^6 LU/ quadriceps observed in untreated control muscle (Figure 1b). Luciferase activity in muscles treated with normal saline (NS), 0.025% or 0.075% bupivacaine was only slightly greater than the activity in the untreated muscles (Figure 1b). Treatment with 0.15%

Figure 1 *Luciferase activity in mouse and rat muscle treated with various agents. (a) ICR mice were intramuscularly injected with 10 μg pBS.RSVL plasmid DNA 5 days after no treatment (none), normal saline (NS), 1.0% etidocaine (E), 2.0% mepivacaine (M), 0.1 M acetic anhydride pH 5.0 (A), 0.1 M sodium bicarbonate pH 9.0 (N), 0.04 mg/ml Notechis scutatus scutatus venom (V), 10 μM cardiotoxin (CT) or 0.75% bupivacaine (B) injections. (b) Balb/c mice were intramuscularly injected with 10 μg pBS.RSVL plasmid DNA 5 days after no treatment (none), normal saline (NS), 0.025%, 0.075%, 0.15%, 0.35% or 0.75% bupivacaine injections. (c) Rat quadriceps were injected with 50 μg pBS.RSVL plasmid DNA 7 days after no treatment (none), normal saline (NS), 0.15%, 0.25%, 0.5% or 0.75% bupivacaine injections. Luciferase activity (in light units, LU, per entire quadriceps muscle) was determined 2 weeks after plasmid DNA injection. Values represent mean luciferase activity per treatment group. The number of muscles (n) analyzed per treatment is indicated in (a) and (b) and is eight in (c). Bars indicate standard error.*

and 0.35% bupivacaine also increased luciferase activity compared with that of either untreated or normal-saline-injected muscle, but the increment obtained from either of these treatments was more variable than that obtained with 0.75% bupivacaine (Figure 1b). Compared with treatment with normal saline, treatment with 0.75% bupivacaine increased luciferase activity approximately 9.6-fold in ICR mice (Figure 1a), 25-fold in Balb/c (Figure 1b), sixfold in C57BL/6 mice (data not shown) and fourfold in *mdx* mice (data not shown).

In rat quadriceps, treatment with bupivacaine caused similar increases in luciferase activity. As rat quadriceps muscle is larger and expresses injected plasmid DNA less efficiently than mouse quadriceps muscle [6], 50 μg pBS.RSVL was injected instead of 10 μg. Luciferase activity in rat muscle treated with 0.50% or 0.75% bupivacaine was 31.8×10^6 and 29.6×10^6 LU/ quadriceps, respectively, which was nearly sixfold greater than the 4.9×10^6 LU/ quadriceps observed in untreated control muscle and approximately twofold greater than the 13.2×10^6 LU/ quadriceps obtained from muscle treated with normal saline (Figure 1c). Rat muscle treated with normal saline had an approximately threefold increase in luciferase activity compared with untreated muscle. Treatment with 0.15% or 0.25% bupivacaine did not substantially increase luciferase activity above treatment with normal saline (Figure 1c).

The time at which plasmid DNA was injected into muscle after bupivacaine treatment also influenced the level of luciferase expression. The optimal interval between 0.75% bupivacaine and pBS.RSVL injection was 7 days for C57BL/6 mice (Figure 2), *mdx* mice (Figure 2) and Sprague-Dawley rats (data not shown), and 5 days for ICR mice and Balb/c mice (data not shown). In all mouse and rat strains tested, injection of plasmid DNA 14 days after 0.75% bupivacaine resulted in similar luciferase activity as non-bupivacaine-treated muscle

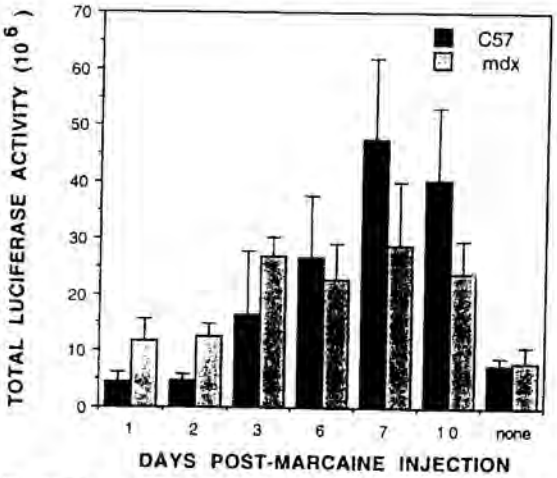


Figure 2 The effect of the time between pBS.RSVL and 0.75% bupivacaine injection on luciferase activity in C57BL/6 or mdx^{lcv} mouse quadriceps. Muscle not treated (none) or treated with 0.75% bupivacaine 1, 2, 3, 6, 7 or 10 days prior to intramuscular injection of 10 μg pBS.RSVL. Luciferase activity (in light units, LU, per entire quadriceps muscle) was determined 2 weeks after plasmid DNA injection. Eight muscles were examined per treatment group. Values represent mean luciferase activity per treatment group. Bars indicate standard error.

(data not shown). The optimal interval between the injection of pBS.RSVL and bupivacaine concentrations less than 0.75% was also 5 to 7 days in all mouse strains tested, and 7 days in rats (data not shown).

Histological analysis and β-galactosidase expression in bupivacaine-treated muscle

As previously described [11–16], muscle treated with 0.75% bupivacaine was nearly devoid of myofibers with extensive phagocytic infiltration 2 to 3 days after bupivacaine injection (Figure 3a). Myotube formation began 4 to 7 days after bupivacaine injection (Figure 3b) and normal muscle histology returned by 19 days after bupivacaine injection (Figure 3c). At bupivacaine concentrations of 0.15% and lower, minimal myofiber damage was observed 2–3 days after injection (Figure 3d), similar to that observed in muscle treated with normal saline. At bupivacaine concentrations between 0.35% and 0.50%, myofiber degeneration was less evident and more variable than that observed in muscle injected with 0.75% bupivacaine. The extent of damage and time required for muscle recovery following the injection of bupivacaine or normal saline was similar in both rat and mouse muscle except that the reformation of myotubes occurred approximately 2 days later in rat muscle (data not shown).

Using conditions which were optimal for luciferase expression, 10 μg pBS.RSVLacZ was injected into ICR mouse quadriceps treated 5 days earlier with 0.75% bupivacaine. Two weeks after plasmid DNA injection (19 days after bupivacaine injection), the quadriceps had mature myofibers reformed, some of which expressed β-galactosidase (Figure 3c). Approximately 60 myofibers per muscle cross-section (≈1.5% of the 4000 myofibers) were stained positively for β-galactosidase (Figure 3e) and these positively stained fibers were observed in all of the cross-sections taken throughout the muscle (data not shown). In untreated control quadriceps, approximately 50 myofibers were β-galactosidase-positive near the plasmid DNA injection site but the number of positively-stained myofibers decreased in cross-sections distant from the site of plasmid DNA injection (data not shown). Two days after plasmid injection, β-galactosidase staining was evident in myotubes (filled arrows, Figure 3b) and mononucleated cells (open arrows, Figure 3b) probably representing satellite cells. In 0.75% bupivacaine-treated muscle injected with pBS.RSVL and assayed for β-galactosidase expression 2 days after plasmid DNA injection, no β-galactosidase staining was observed (data not shown).

Stability of reporter gene expression in bupivacaine-treated muscle

ICR mouse quadriceps were injected with pBS.RSVL 5 days after 0.75% bupivacaine injection and then assayed for luciferase activity at varying times afterwards. Mean, total luciferase activity ($\times 10^6$ LU/ muscle; n=8) was 123 ± 21.6, 363 ± 123, 10.6 ± 4.9 and 12.8 ± 6.6 at 2 weeks, 1 month, two months and 4 months after pBS.RSVL injection, respectively (Figure 4). The nearly threefold increase in luciferase activity from

Figure 3 Photomicrographs of bupivacaine-treated ICR mouse quadriceps muscle. Cross-section of ICR mouse quadriceps 3 (a), 7 (b) and 19 days (c) after the injection of 0.75% bupivacaine. Cross-section of ICR mouse quadriceps 3 days after the injection of 0.15% bupivacaine (d). β-Galactosidase expression was also visualized in 0.75% bupivacaine-treated muscle 2 days (b) and 14 days (c, e) after the injection of 10 μg pBS.RSVLacZ (7 and 19 days post-bupivacaine injection, respectively). In (b), open arrows indicate mononucleated cells whereas filled arrows indicate myotubes expressing β-galactosidase. Hematoxylin and eosin staining was done for cross-sections shown in (a) and (d) whereas (b), (c) and (e) were stained only for β-galactosidase positive fibers. Magnifications: ×100 for (a) and (d), ×40 for (b) and (c), and ×6.25 for (e).

2 weeks to 1 month after plasmid injection was similar to increases observed in untreated muscle [9]. However, by 2 months after plasmid injection the luciferase levels had decreased 12-fold from that observed at 2 weeks (Figure 4), whereas untreated muscle typically showed a threefold increase over the same time period [9].

Transient expression was also observed with bupivacaine-treated mouse muscle injected with pBS.RSVLacZ. Two weeks after pBS.RSVLacZ injection, approximately 60 β-galactosidase-expressing myofibers were observed per muscle cross-section (Figure 3e). But by 2 months after pBS.RSVLacZ injection no more than one β-galactosidase-expressing myofiber could be observed per cross-section (data not shown).

Quantitative polymerase chain reaction (PCR) analysis was performed to determine the amount of pBS.RSVL in the untreated and 0.75% bupivacaine-treated quadriceps muscle of ICR mice at varying times after plasmid injection. At 2 weeks after pBS.RSVL injection, a greater amount of plasmid DNA was present

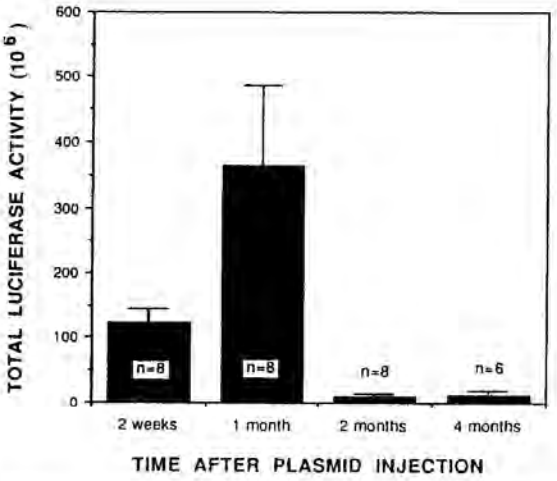


Figure 4 Stability of luciferase expression in mouse muscle treated with 0.75% bupivacaine. ICR mouse quadriceps were given intramuscular injections of 10 μg pBS.RSVL plasmid DNA 5 days after treatment with 0.75% bupivacaine. The muscle was assayed for luciferase activity 2 weeks, 1 month, 2 months and 4 months after plasmid DNA injection. The number of muscles (n) examined per treatment group is indicated in the figure. Values represent mean luciferase activity per treatment group. Bars indicate standard error.

in the untreated than in the bupivacaine-treated muscle (Figure 5). At 1 month after plasmid injection, bupivacaine-treated and untreated muscle contained similar amounts of plasmid DNA despite the large increase in luciferase activity in bupivacaine-treated muscle at this time (Figures 4 and 5). The amount of plasmid DNA in the bupivacaine-treated muscle 2 months after injection was similar to the amount in bupivacaine-treated muscle 1 month after injection despite the large decrease in luciferase activity observed over this time (Figures 4 and 5).

Discussion

Luciferase activity after pBS.RSVL intramuscular injection increased substantially by treating muscle with 0.75% bupivacaine prior to plasmid DNA injection. In mice, luciferase activity increased, depending on the strain, four- to 40-fold above the levels obtained in untreated control muscle (Figures 1 and 2). In Sprague-

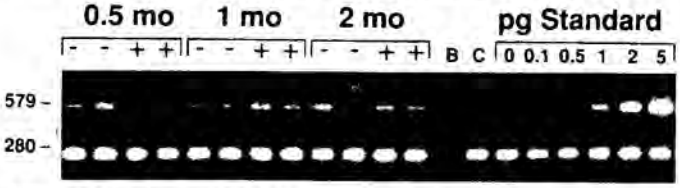


Figure 5 The amount of pBS.RSVL in mouse quadriceps determined by semiquantitative PCR. Two mouse quadriceps muscles were not treated (–) or treated (+) with 0.75% bupivacaine 5 days prior to the injection of 10 μg pBS.RSVL plasmid DNA. At 0.5, 1 and 2 months (0.5, 1 and 2 mo, respectively) after pBS.RSVL injection, the muscle was removed and DNA extracted. Following DNA extraction and quantification, 2 μg of tissue DNA was subjected to semiquantitative PCR. The experimental amount of pBS.RSVL resulted in a 579 base pair amplification product whereas the internal standard was 280 base pairs. Amplification products obtained from muscle not injected with pBS.RSVL are shown in lanes B and C. Standard amounts of pBS.RSVL in 2 μg of muscle DNA are also shown. Internal standard (2 pg) was included in all samples except lane B.

Dawley rats, 0.75% bupivacaine treatment increased luciferase activity six- and twofold above the levels in untreated and normal-saline-injected muscle, respectively (Figures 1 and 2). Whereas the other agents tested selectively damaged myofibers and enabled effective regeneration, 0.75% bupivacaine treatment increased luciferase activity the most. It has previously been reported that injecting 50–100 μl of hypertonic sucrose 15–30 min prior to plasmid DNA injection greatly decreased the variability and slightly improved the mean level of luciferase expression in C57BL mouse tibialis anterior muscle [27]. Similar results were obtained when 0.1 M sodium-phosphate-buffered saline pH 7.3 was used instead of hypertonic sucrose [27]. The 0.75% bupivacaine treatment clearly enhanced expression beyond that observed for hypertonic sucrose or normal saline, particularly in Balb/c and ICR mouse muscle.

The ability for bupivacaine and cardiotoxin to increase the expression of intramuscularly injected plasmid DNA has been previously reported [28] (H. Davis and R. Whalen, personal communication). For immunization against HIV, plasmid DNA has also been intramuscularly injected into mouse muscle treated with bupivacaine 2 days prior to plasmid injection [29]. Our results suggest that optimal luciferase expression was obtained when the plasmid DNA was injected 5 to 7 days after bupivacaine injection (Figure 2); a time when a substantial number of muscle cells have begun to recover from the effects of bupivacaine (Figure 3). A similar experience was observed in regenerating liver which showed long-term persistence and expression of the transferred gene *in vivo* following partial hepatectomy preceded by asialoglycoprotein/polylysine-mediated gene transfer [30].

As in non-treated muscle, luciferase activity in bupivacaine-treated muscle increased when assayed 4 weeks after plasmid DNA injection compared with that assayed at 2 weeks (Figure 4). However, in marked contrast to non-treated muscle, luciferase activity in bupivacaine-treated muscle decreased over 30-fold when assayed 2 or 4 months after plasmid DNA injection compared to levels obtained 1 month after plasmid injection (Figure 4).

The increased luciferase activity in bupivacaine-treated muscle may be due to either increased plasmid DNA uptake or increased expression of the plasmid DNA via transcriptional or translational mechanisms. The quantitative PCR analysis suggests bupivacaine-treated muscle did not take up significantly more plasmid DNA and that the increased luciferase activity was mostly due to increased expression. Peaking of luciferase activity at 1 month after pBS.RSVL injection may be related to transient increases in fetal and embryonic myosin expression observed following cardiotoxin injection in mammalian muscle [25]. Similar changes in myosin isoform expression have also been observed after intramuscular bupivacaine injection [31]. Increased levels of slow myosin were also observed in regenerating rat soleus muscle following notexin treatment [26]. Therefore, the transient increase in

luciferase expression observed in regenerating muscle may result from improved expression of the plasmid DNA for approximately 1 month after bupivacaine injection.

Semi-quantitative PCR analysis is not sufficiently precise to exclude the possibility that increased luciferase activity in bupivacaine-treated muscle was due in part to increased plasmid uptake. Also, the plasmid DNA present in the total muscle extract may not be representative of nuclear plasmid DNA. The greater luciferase activity obtained when pBS.RSVL was injected 5 to 7 days after bupivacaine treatment compared with later time points (Figure 2) can be best explained by increased plasmid DNA uptake. The β-galactosidase expression observed in mononucleated cells and myotubes 2 days after pBS.RSVLacZ injection in regenerating muscle (Figure 3b) was consistent with previous observations of naked plasmid DNA expression by myoblasts and myotubes in culture [10]. The presence of developing myofibers in regenerating muscle may increase the muscle's ability to take up plasmid DNA, either by allowing better distribution of the plasmid DNA throughout the muscle, or the mitotically active developing myofibers more effectively take up plasmid DNA compared to mature fibers. In addition, plasmid DNA uptake could be increased by the 20–40 mV membrane depolarization which occurs for more than 3 days after bupivacaine administration *in vivo* [32].

In this study, the levels of luciferase activity achieved from 10 μg pBS.RSVL injected into muscle were greater than those obtained when it was transfected into cultured cells. For example, total luciferase activity was 182×10^6 LU in Balb/c mouse muscle 2 weeks after plasmid injection and was 363×10^6 LU in ICR muscle 1 month after plasmid injection. In comparison, the transfection of 10 μg pBS.RSVL under optimal conditions yielded a total luciferase activity of $10–20 \times 10^6$ LU in 3T3 cells and $100–200 \times 10^6$ LU in primary rat muscle cells [4, 10, 33]. Therefore, in muscle treated with 0.75% bupivacaine, the plasmid DNA was more efficiently expressed following intramuscular injection than in cultured cells tested under optimal conditions.

For clinical applications, the intramuscular injection of plasmid DNA could potentially restore normal muscle function in intrinsic muscle diseases, secrete a hormone or growth factor, clear a circulating toxic metabolite, or vaccinate against a specific disease. The fact that bupivacaine treatment causes extensive short-term muscle damage and resulted in a transient increase in reporter gene expression suggests that it would be best used for inducing a more effective immune response in vaccination protocols. Inflammation, which accompanies bupivacaine treatment, may also aid the immunization process. Previous studies in non-human primates and rats have found low levels of foreign gene expression after intramuscular plasmid DNA injection [6, 7]. These low expression levels were thought to result from connective tissue barriers which impeded perfusion of the injected plasmid DNA into the muscle. As foreign gene expression data obtained in rats correlates well to that observed in non-human primates [7], the use of bupivacaine treatment prior to plasmid DNA injection may be critical for clinically successful vaccination by intramuscular plasmid DNA injection.

Materials and methods

Reporter genes

The pBS.RSVL luciferase expression plasmid was prepared as follows. The plasmid pBluescript SK(-) (Stratagene) was digested with *Pvu*II to remove the entire multiple cloning site (MCS) and ligated to the 1812-bp *Nru*I*Sca*I fragment from pcDNA I (Invitrogen) containing the cytomegalovirus (CMV) promoter, MCS and simian virus 40 (SV40) poly(A) and splice site. The clone in which the CMV promoter was oriented adjacent to the ColE1 ori from pBluescript SK(-) was chosen and named pBlueCMV. The CMV promoter in pBlueCMV was removed by digesting pBlueCMV with *Nde*I/*Hin*dIII and replaced with the *Nde*I/*Hin*dIII fragment from pRSVL [1] containing the entire RSV promoter to form pBlueCERSV. The *Hin*dIII/BamHI fragment from pJD206 containing the luciferase cDNA [34] was inserted into the MCS of pBlueCERSV to form pBlueCERSVLux. The CMV/RSV fusion promoter in pBlueCERSVLux was removed by *Nru*I/*Hin*dIII digestion and replaced with a *Spe*I (Klenow)/*Hin*dIII fragment containing the SV40 early promoter to yield pBS.RSVL.

The pBS.RSVLacZ β-galactosidase expression plasmid was constructed by placing the *Hin*dIII/*Bam*HI fragment from pSDKLacZpA (gift of J. Rossant) containing the *E. coli lac*Z gene [35] into the *Hin*dIII/*Bam*HI fragment of pBlueCERSV (see above), which contained the RSV promoter and SV40 intron/poly(A) signal. All plasmid DNA was purified by alkaline lysis and two CsCl gradients as previously described [7].

Injection of bupivacaine and other compounds into rodent muscle

C57BL/6, Balb/c and ICR mice strains and Sprague Dawley rats were obtained from Harlan Sprague Dawley (Indianapolis, IN, USA). The *mdx*^{3cv} strain [36, 37] was obtained from V. Chapman.

Bupivacaine hydrochloride (Marcaine, Abbott Laboratories, Chicago, IL, USA) was injected into muscle using previously established protocols [11–16]. Briefly, 100 μl bupivacaine, diluted in normal saline, was injected into the quadriceps of anesthetized mice. For rat injections 600 μl bupivacaine was used. Mice were anesthetized using either 2.5% Avertin or methoxyflurane (Metofane, Pitman-Moore, Inc., Mundelein, IL, USA) and rats were anesthetized using ketamine/xylazine as previously described [6]. Concentrations of bupivacaine injected are given in figure legends. The same injection procedure was used for the injection of etidocaine hydrochloride (Duranest, Astra, Westborough, MA, USA), mepivacaine hydrochloride (Polocaine, Astra, Westborough, MA, USA), acetic anhydride [24], sodium bicarbonate [24], *Notechis scutatus scutatus* venom (Notexin, Sigma,

St. Louis, MO, USA) [26] and cardiotoxin (Latoxan, Rosans, France) [25] into muscle.

Plasmid DNA injection and analysis of reporter gene expression

Injection of plasmid DNA into mouse and rat quadriceps followed previously described methods [6]. The plasmid DNA was diluted in 100 µl normal saline for mouse muscle injections and in 400 µl for rat muscle injections. Specific amounts of DNA injected into each muscle are given in the figure legends. Determinations of luciferase and β-galactosidase expression were as previously described [1, 6], and are presented as the total luciferase activity obtained per entire quadriceps muscle.

Quantitative PCR analysis

Semi-quantitative PCR analysis was as previously described [4], except that the reaction fluid contained 2 µg tissue DNA in a total reaction volume of 100 µl. Also 0.2 pg internal control luciferase template was used instead of 0.1 pg. A total of 10 µl of the final PCR reaction fluid was separated by electrophoresis in a 2.5% agarose gel [2.0% metaphor agarose (FMC) and 0.5% electrophoresis grade agarose (BRL)], and stained with 0.5 µg/ml ethidium bromide.

Note added in proof

Recent publications showed similar results with regard to foreign gene expression in regenerating skeletal muscle [38, 39].

Acknowledgments

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High expression of naked plasmid DNA in muscles of young rodents

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There is a time window at 2 weeks of age for achieving very high levels of foreign gene expression from the intramuscular injection of naked plasmid DNA in mice and rats. The highest expression, over 1 μg of luciferase protein/muscle, was obtained in Balb/C mice using constructs containing the CMV promoter, a chimeric intron and the luc+ luciferase gene. Approximately 50% of the myofibers were intensely blue following the intramuscular injection of a β-galactosidase expression vector in 2 week old Balb/C mice. The effects of age, mouse strain and construct were multiplicative, resulting in >1000-fold greater luciferase and ~20-fold more β-galactosidase-positive cells. These high levels of expression were unstable and were not observed in larger animals (dog, rhesus monkey). These results indicate that enormous levels of foreign gene expression can be obtained in muscle with naked DNA *in vivo* and will enable the temporary effects of gene function and expression in rodent muscle to be expeditiously studied.

INTRODUCTION

Several methods of gene transfer into muscle are under development for laboratory studies and gene therapy (1–3). These include direct methods in which the gene is delivered to muscle *in vivo* and *in situ* and indirect methods in which the gene is delivered to myoblasts in culture that are then transplanted (4–7). Direct methods are favored because of their inherent simplicity and the inefficiency of myoblast transplantation (8–11).

Both viral and non-viral vectors have been used for direct gene transfer into muscle. The viral vectors have been derived from adenoviruses (12), herpes simplex virus (13), retroviruses (14–16) and more recently adeno-associated viruses (17,18). While molecular conjugates (e.g., polylysine complexes) and cationic lipids have been used to deliver genes directly into other tissues (19,20), they have not been generally useful in muscle tissue *in vivo*. Naked plasmid DNA (pDNA) remains the most efficacious non-viral method in skeletal, diaphragmatic, and cardiac muscle to date (21–24). The mechanism of DNA uptake is unknown, although we and others have postulated an active cellular uptake process (25–27).

A variety of factors have been shown to affect the levels of foreign gene expression from intramuscularly injected pDNA. Muscle regeneration induced by myotoxic agents enabled higher levels of expression (28,29). Expression can also be aided by improved distribution of the pDNA which has been accomplished by preinjection of muscles with large volumes of hypertonic solutions, polymers, or by improved injection technique (26,30,31). Several studies have shown that newborn animals can express naked plasmid DNA (32–35). In carp, plasmid expression of chloramphenicol acetyl transferase (CAT) in muscle was greater in young than old fish (33). Plasmid expression was deemed efficient after injection into *Xenopus* tadpole muscle (34). Of particular note was the increased CAT expression from the SV40 promoter in 4–6 week old mice as compared with older mice (35). Other studies have shown the importance of the promoter and construct. Generally, the viral promoters RSV and CMV enable higher levels of expression than mammalian promoters (32,36). Luciferase expression from a CMV immediate-early promoter with a chimeric intron was ~3-fold greater than from a RSV promoter (36). Further studies by the same group showed that replacement of the kanamycin gene for the ampicillin gene, removal of SV40 origin of replication, modification of the plasmid backbone and improved transcription terminators brought about very large increases in luciferase expression (37).

The present report extends these previous studies and demonstrates exceptionally high levels of foreign gene expression from refined plasmid vectors in 2 week old mouse and rat muscles. Preliminary studies are presented in dogs and non-human primates. The implications for laboratory studies and gene therapy are discussed.

RESULTS

Expression is optimal at 2 weeks of age

Ten μg of pCILuc in 50 μl of normal saline were injected into the quadriceps of mice and rats at different ages and the luciferase activities were assayed in the entire muscle after 1 week (Fig. 1). In all three mouse strains, the greatest luciferase levels were obtained at 2 weeks of age. The mouse 2 week levels were 30- to 120-times higher than the 8 week levels. Injections into 20 week old Balb/C mice yielded 1200-times less luciferase expression on average than in 2 week old mice. In the newborn mice, the lower levels of expression are possibly due to the injection fluid not being well-retained in the very small muscles.

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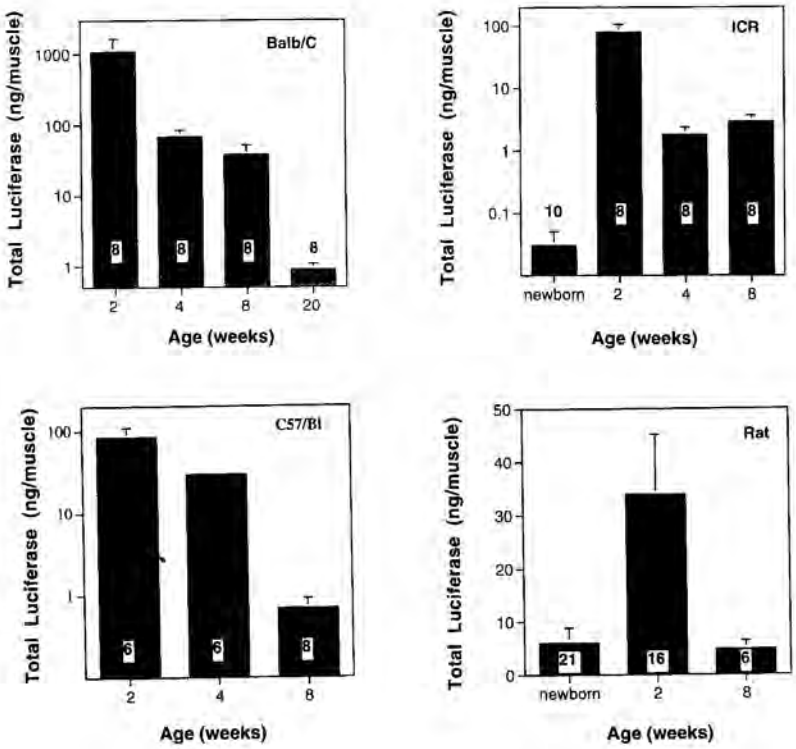


Figure 1. Mean luciferase levels in muscles 1 week after 10 µg of pCILuc was injected into the quadriceps of Balb/C, ICR, and C57/Bl mice and Sprague–Dawley rats at different ages. Bold numbers indicate the number of muscles assayed at each time point. Bars indicate the standard error.

Substantially higher levels of expression were obtained in Balb/C mice than in the other two mouse strains (Fig. 1). In 2 week old mice, the 1080 ng of average luciferase expression in Balb/C mice was ~13 times higher than the ~80 ng in ICR and C57/Bl mice.

High levels of luciferase expression were also obtained in the tibialis anterior (TA) muscle of 2 week old ICR mice. The mean of 12.0 ng of total luciferase in the TA muscles (± 4.0, $n = 8$) is 6.6 times less than the mean in quadriceps muscles of 2 week old ICR mice. The TA muscle is ~5 times smaller than the quadriceps muscle so that the amounts of luciferase expression per weight of muscle tissue are similar in the two muscles.

Similar results were also obtained in Sprague–Dawley rats following the injection of 100 µg of pCILuc in 200 µl. In 2 week old rats, an average of 34 ng of luciferase was obtained. The 2 week average was seven-times higher than the 8 week average (Fig. 1). The lower expression in newborn rats is genuine because their quadriceps muscles are of sufficient size to retain the injection fluid.

The relationship between the amount of pDNA and the level of luciferase expression was determined by assaying luciferase levels 1 week after 1, 3, 10 and 50 µg of pCILuc were injected into the quadriceps muscles of 2 week old ICR mice (Fig. 2). The dose response curve indicates a logarithmic relationship between mean luciferase levels and the amount of DNA injected.

Given that the high levels of expression in the younger animals may be due to their decreased extracellular matrix, injections were also done in homozygous 129/ReJ-*Lama2*^{dy} adult mice that are genetically deficient in M-laminin (38). Seven days after 10 µg of pCILuc were injected into the quadriceps of 8 week old dystrophic 129/ReJ-*Lama2*^{dy} mice the mean luciferase level was

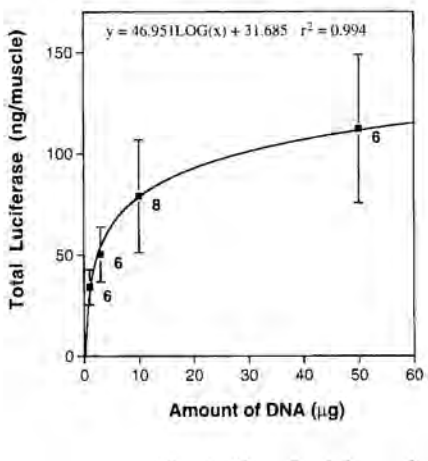


Figure 2. Dose-response curve. Mean luciferase levels in muscles 1 week after various amounts of pCILuc in 50 µl of normal saline was injected into the quadriceps of 2 week old ICR mice. Bold numbers indicate the number of muscles assayed for each quantity of DNA. Bars indicate the standard error.

8.6 ng/muscle (± 4.9, $n = 8$) which is 5.3 times greater than the mean of 1.6 ng/muscle (±1.9, $n = 8$) in normal heterozygous littermates.

Histological studies

The β-galactosidase reporter system was also used to determine the effect of age on expression. One week after 10 µg of pCILacZ in 50 µl of normal saline was injected into the quadriceps of 2 week old ICR mice, the muscles were examined histologically for β-galactosidase expression. Approximately 50% of the

myofibers were intensely blue (Fig. 3A), which is at least one order of magnitude greater than the percent of positive myofibers our laboratory has previously obtained in older mice. A large number of β-galactosidase-positive myofibers were also obtained in 2 week old rats (Fig. 3B, C). No blue myofibers are found following injection with pCMVLux.

The quadriceps muscles of 2 week old *mdx* mice were injected with 400 μg of pRSVBecker DNA and immunohistochemically stained for dystrophin expression 1 week later (Fig. 3D). The mean number of dystrophin positive fibers per cross-section was 142 (± 18, 4–6 cross-sections per muscle, six muscles). This number is about three times greater than we had previously obtained in older *mdx* mice also using 400 μg of pRSVBecker. The quadriceps of control 2 week old *mdx* mice injected with pUC19 DNA did not contain any positive fibers. *Mdx* muscles injected with 400 μg of pCIBecker inexplicably showed only a few dystrophin-positive fibers.

Fluorescently labeled DNA was injected into 2 week old and adult mice. Extracellular and intramyofiber staining were greater in the 2 week old mice, consistent with improved distribution through the extracellular matrix and cellular uptake (Fig. 4A). Most interestingly, the staining pattern in some of the myofibers in the 2 week animals was diffuse which suggests pDNA entry into the sarcoplasm (Fig. 4B) (21). Adult myofibers did not exhibit this diffuse pattern (data not shown). Muscles from both the young and older mice also contain curvilinear and punctate staining which may reflect DNA entry into T tubules (25). Electron microscopic studies are necessary to definitively determine the exact sub-cellular location of the injected DNA.

Different promoters

The luciferase expression of various constructs containing different promoters and luciferase genes were compared in 2 week old ICR mice (Table 1). The two pCI-based constructs, pCILuc and pCILuc2 yielded the highest levels of expression. A comparison of luciferase expression from pCILuc and pBS.CMVLuc indicates that the pCI elements enabled 8-fold more expression. A comparison of luciferase expression from pBS.CMVLuc and pBS.CMVLux indicates that the Luc gene enabled 2.5-times more expression than the Lux gene. These differences are similar to those obtained following transient transfection with cationic lipids into 3T3 cells in culture (data not shown). Finally, a comparison of pBS.CMVLux and pBS.RSVLux indicates that the RSV promoter enabled approximately two times more luciferase expression.

The luciferase expression of the various constructs in 2 week old ICR mice was compared with their expression in older ICR mice (Table 2). For all the constructs, the greatest expression was obtained at 2 weeks of age; the least expression was obtained at 8 weeks of age. The ratio of 2–8 week old expression was greatest for pBS.RSVLux and smallest for SV40. In C57/Bl mice, injections of pCILuc also yielded higher levels of expression in 2 week old mice (Table 3). However, injections of pSV40Lux DNA yielded similar levels in 2 and 4 week old mice but less in 8 week old mice (Table 3).

The muscle creatine kinase promoter was evaluated in young and old Balb/c mice. One week after injection of pBS.MCKLuc, the mean luciferase level in 2 week old mice was 12.4 ng/muscle (±4.9, n = 8), ~90 times greater than the mean of 0.14 ng (±0.13, n = 8) in 8 week old mice.

Table 1. Total mean luciferase levels 1 week after injection of various plasmids into 2 week old ICR quadriceps muscle (n = 8)

Injected plasmid	Total luciferase (ng)	Standard error	% compared with pCILuc
pCILuc	79.04	27.87	100
pCILuc2	101.95	48.88	128
pBS.CMVLuc	9.65	2.91	12
pBS.CMVLux	3.76	0.99	5
pBS.RSVLux	8.51	2.32	11
pSV40Lux	2.00	0.61	2.5

Table 2. Total mean luciferase levels 1 week after injection of different plasmids into 2, 4 and 8 week old ICR quadriceps muscle

Plasmid	2 week LUX (ng)	4 week LUX (ng)	Ratio	8 week LUX (ng)	Ratio
pCILuc	79.04	17.9	4.42	2.90	27.2
pBS.CMVLuc	9.65	0.33	29.1	0.11	86
pBS.RSVLux	8.51	0.57	14.9	0.08	104.3
pSV40Lux	2.00	0.30	6.8	0.13	15.1

Ratio indicates the relative expression from the 2 week old mice compared with that of either 4 or 8 week old mice.

Table 3. Total mean luciferase levels 1 week after injection of different plasmids into 2, 4 and 8 week old C57/Bl quadriceps muscle

Plasmid	2 week LUX (ng)	4 week LUX (ng)	Ratio	8 week LUX (ng)	Ratio
pCILuc	84.3	29.7	2.83	0.69	122.2
pSV40Lux	0.79	0.88	0.90	0.06	13.2

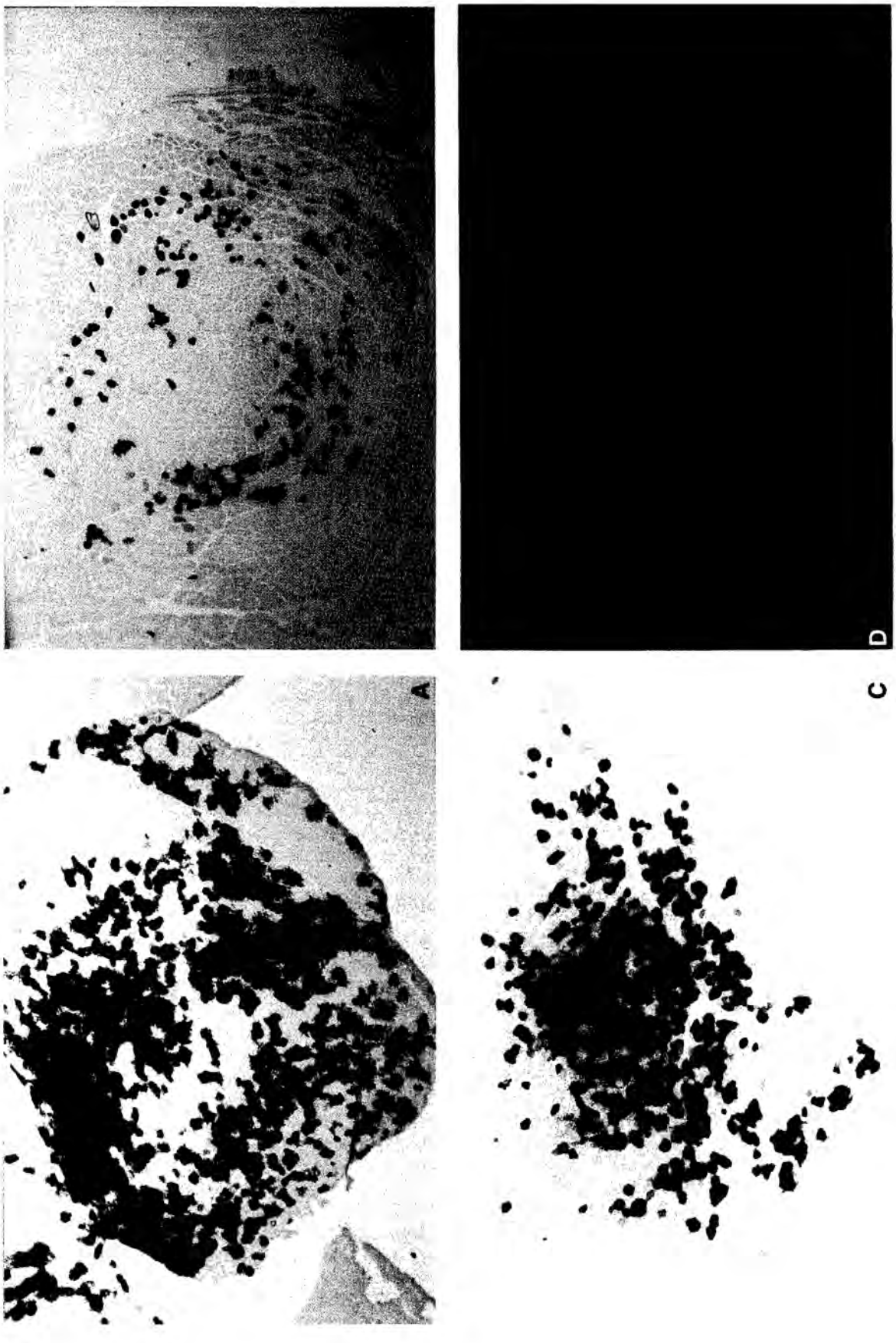
Ratio indicates the relative expression from the 2 week old mice compared with that of either 4 or 8 week old mice.

Stability of luciferase expression

The stability of luciferase expression was determined by assaying the levels in excised quadriceps muscles at various times after the muscles in 2 week old ICR mice were injected with different expression constructs (Fig. 5A). The most time points were analyzed for the pCILuc construct for which the luciferase levels decreased ~250-fold from 1–2 weeks after injection (Fig. 5A). Thereafter, luciferase expression persisted for at least 22 weeks. Luciferase levels also decreased from 1 to 8 weeks after injection of the pBS.CMVLux and pBS.RSVLux constructs (Fig. 5A).

In 8 week old ICR mice, luciferase expression decreased 20-fold from 1 to 8 weeks after injection of pCILuc (Fig. 5B). Yet, luciferase expression increased from 1 to 8 weeks after injection of pBS.CMVLux or pBS.RSVLux (Fig. 5B).

Similar trends were also observed in Balb/C mice (Fig. 5C, D). In 2 week old Balb/C mice, luciferase expression decreased substantially from 1 to 2 and 8 weeks after injection of pCILuc (Fig. 5C). However in 20 week old Balb/C mice, luciferase expression increased from 1 to 8 weeks after injection of pCILuc, pBS.CMVLux, or pBS.RSVLux.


A
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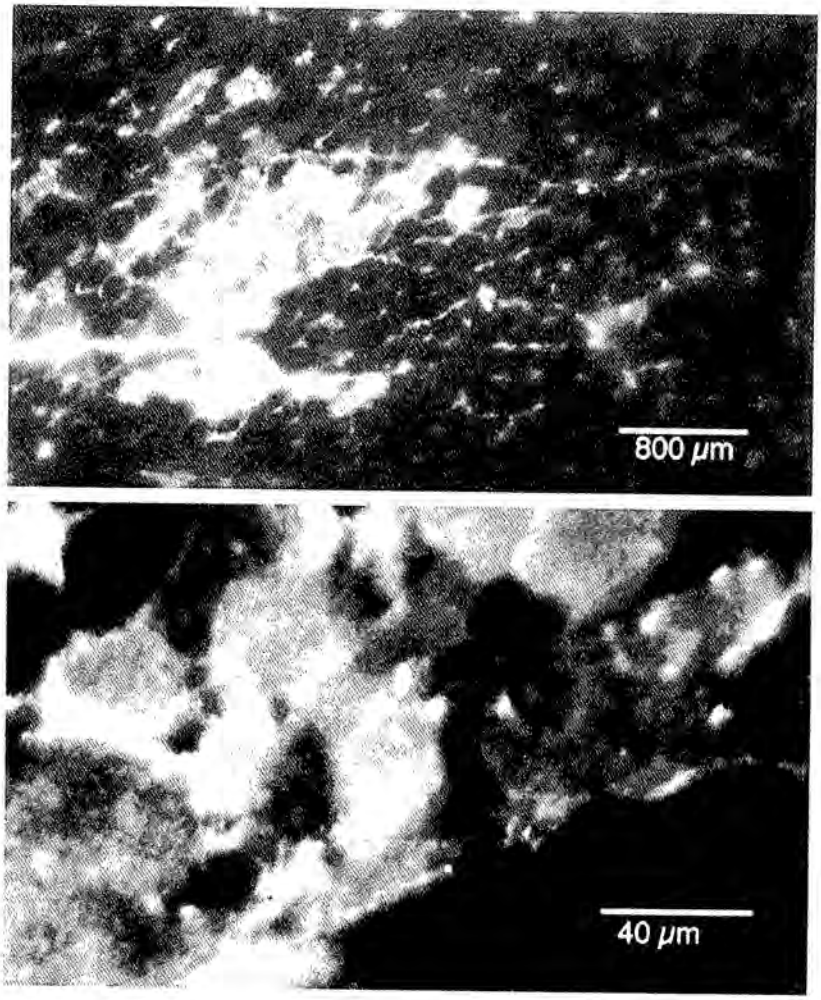

Figure 4. Fluorescent pDNA was injected into quadriceps muscles of 2 week old Balb/C mice (10 μg per muscle). Mice were sacrificed and muscle excised 1, 4 and 20 h after injection. (**A**) 1 hour after injection; (**B**) 4 h after injection.

Table 4. Total mean luciferase levels 1 week after 2 week old monkeys (two monkeys, four muscles) were injected with 1 mg of pCILux (in pg ± standard error)

Muscle type	Total LUX (pg)	pg LUX/g muscle
tibialis anterior (TA)	47 ± 31	857 ± 500
biceps	77 ± 57	865 ± 527
rectus femoris (quadriceps)	186 ± 112	1356 ± 631

In contrast to the unstable expression in 2 week old untreated mice, expression persisted in 2 week old immunosuppressed mice. Specifically, 10 μg of pCILux were injected into 2 week old Balb/C mice receiving FK506. The mean luciferase level at 1 month after pDNA injection ($n = 20$) was 59% of the mean at 1 week after injection ($n = 10$). In 2 week old control animals that did not receive FK506, the mean luciferase activity at 1 month was 4% of the mean at 1 week after injection.

Luciferase expression in dog and monkey muscle

Luciferase activity was assayed 1 week after various muscles in puppies of different ages were injected with 200 or 1000 μg of pCILux in one ml of normal saline. Overall the luciferase levels were substantially less than those in rats (Fig. 6). The maximum levels were obtained in 4 week old puppies and in the TA and biceps muscles which are smaller than the quadriceps. Whereas the highest levels of total muscle luciferase in the dogs were 2–6-fold less than those in C57/Bl and ICR mice and rats, the efficiencies of expression in terms of ng luciferase/μg pCILux injected were ~62- and ~2.7-fold less than that in the mice and rats, respectively.

Figure 3. Quadriceps muscles were histochemically stained for β-galactosidase activity 1 week after intramuscular injection of 10 μg of pCILacZ in 2 week old ICR mice (**A**) or 100 μg of pCILacZ in 2 week old rats (**B** and **C**) or immunohistochemically stained for dystrophin expression 1 week after injection of pRSVBecker in 2 week old *mdx* mice (**D**). The sections shown are representative of the results obtained in four different animals. Magnifications: A, 40×; B, 15×; C, 40×; and D, 25×.

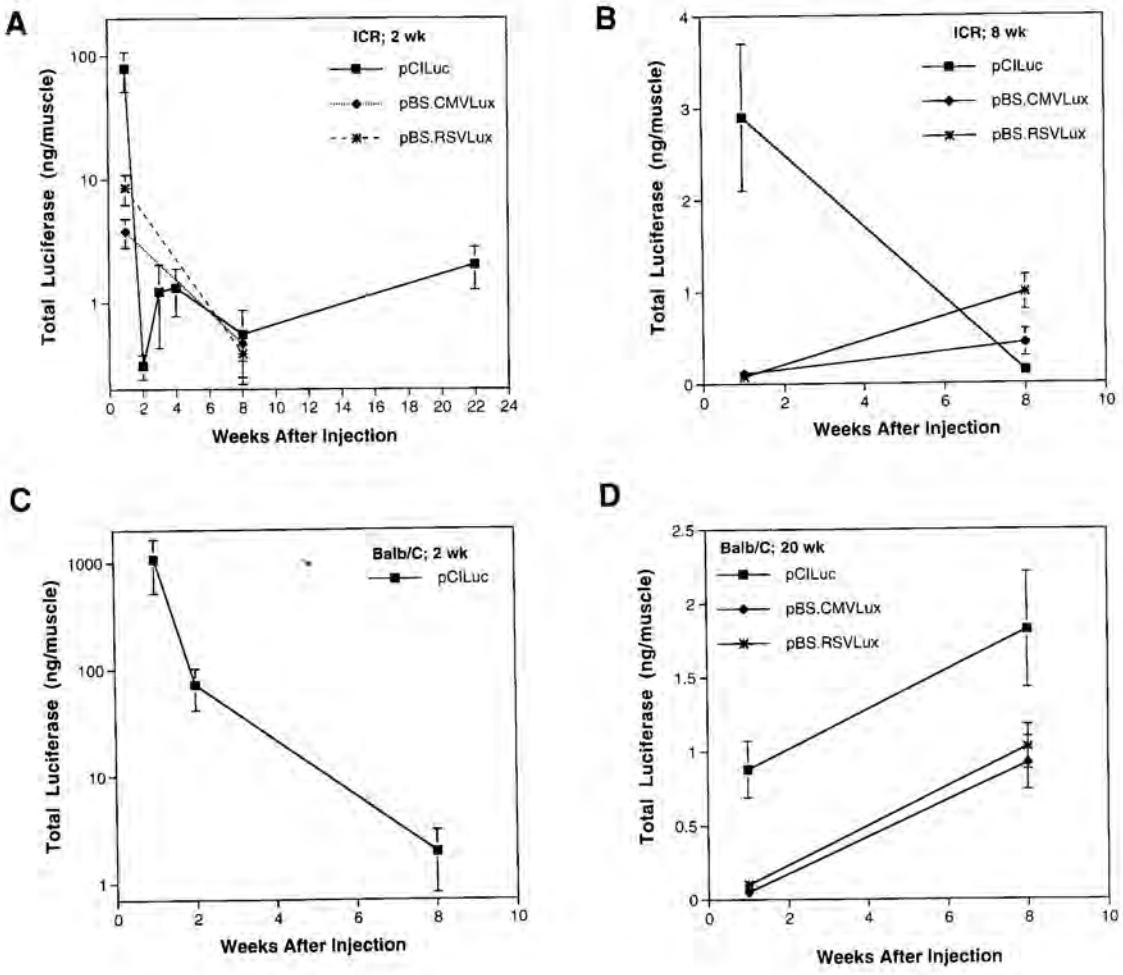

Figure 5. Mean luciferase levels at various times after 10 μg of luciferase expression constructs were injected into the quadriceps of 2 week old ICR mice (**A**), 8 week old ICR mice (**B**), 2 week old Balb/C mice (**C**), and 20 week old Balb/C mice (**D**). Error bars indicate the standard error. Six to 10 muscles were assayed for each time point.

Similar injections and analyses were done in 2 week old monkeys except 40–180 mg of muscle were obtained on open biopsy at the site of injection (Table 4). One week after injection with 1 mg of pCILux in 0.5 ml of normal saline, the muscles contained substantially less luciferase activity than did the rodents. In terms of luciferase activity per mg of muscle tissue, the primate results were similar to that in 2 week old puppies but ~10-fold less than that in 4 week old puppies.

DISCUSSION

These results indicate that there is a time window at 2 weeks of age for achieving very high levels of foreign gene expression from intramuscularly injected pDNA. This time window was observed with several different constructs, which contained either the CMV, RSV, SV40, or MCK promoter. It also occurred in three different mouse strains and in rats. The highest expression, over 1 μg of luciferase protein/muscle, was obtained in Balb/C mice using constructs containing the CMV promoter, chimeric intron, and the luc+ luciferase gene. Of note was that the effects of age and construct were multiplicative. We have recently shown that very high levels of expression in hepatocytes can be obtained when pDNA in hypertonic solutions is injected into the portal vein (39). The highest efficiencies of expression in terms of ng of luciferase protein/μg of pCILuc delivered were comparable in 2 week old muscle (~100 ng/μg) and liver (~170 ng/μg) (Zhang *et al.*, in preparation). These muscle and liver results indicate that enormous levels of foreign gene expression can be obtained with naked DNA *in vivo*.

The only deviation to this time window at 2 weeks of age was the expression of pSV40Lux in C57/Bl mice (Table 3). Luciferase expression in 2 and 4 week old mice were similar but substantially less in 8 week old animals. The similar levels in 2 and 4 week old mice were specific to the injection of pSV40Lux in C57/Bl mice. It was observed neither with the pCILuc vector in C57/Bl mice (Table 3) nor with pSV40Lux in ICR mice (Table 2). Our results with pSV40Lux in C57/Bl mice were similar to the previously published results of Wells and Goldspink who observed higher expression of CAT from the SV40 promoter in 4 week old than from 2 week old C57/Bl mice (35).

The reason for greater expression in 2 week old rodents is open to speculation. Since the mechanism of pDNA uptake in muscle is not known, definitive conclusions cannot be reached. The larger number of β-galactosidase-positive cells is consistent with increased cellular pDNA uptake and a greater number of transfected myofibers. The diffuse pattern of intramyofiber

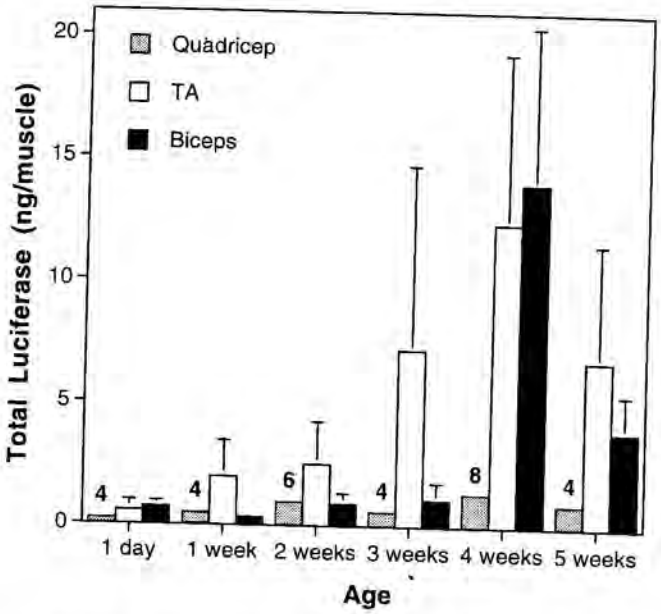

Figure 6. Mean luciferase levels 1 week after various muscles in puppies of different ages were injected with 200 or 1000 μg of pCILux in 1 ml of normal saline. The luciferase activities in muscles injected with different amounts of plasmid DNA were averaged together because they were not significantly different. Half of the 1 day old pups were injected with either amount. Two of the six muscles in the 4 week old pups were injected with 1000 μg. All of the 1 week old and 2 week old pups were injected with 1000 μg. All of the 3 and 5 week old pups were injected with 200 μg. Bold numbers indicate the number of muscles assayed for each muscle group at each time point. Bars indicate the standard error.

staining observed after intramuscular injection into 2 week old (but not adult) mice may be due to a greater ability for cellular DNA uptake in the younger muscle. Also, Southern blot analysis indicated that the amount of pDNA 1 week after injection was greater in 2 week old than adult mice (data not shown). The greater amount of pDNA associated with the muscle may also result from a better delivery of the pDNA throughout the young muscle because it contains less extracellular matrix to limit its distribution (25,40). The greater expression in dy/dy mice which have a disrupted extracellular matrix is consistent with this hypothesis. The greater infectivity of adenoviral or herpes virus vectors in younger mouse muscles has been explained by a decreased limitation by the extracellular matrix (13,41,42).

On the other hand, the increased expression could be the result of increased transcriptional activity of the promoters. The cellular milieu of myofibers in 2 week old animals may contain increased amounts of rate-limiting transcriptional factors that are common to the four promoters studied. Increased transcriptional activity may not only explain the increased luciferase levels but also the increased number of β-galactosidase-positive cells. Previous studies using flow cytometry have suggested that enhancers increase the likelihood of expression from a transfected gene and not necessarily the amount of expression per cell (43). Similarly, the greater number of β-galactosidase-positive cells could result from an increased probability that the transfected gene is transcriptionally active in young muscle. Also, the small myofibers in the younger muscle may concentrate the β-galactosidase and lower the threshold for detecting X-gal staining. However, greater expression in young mice was also observed when the MCK promoter was used. The fact that this promoter is more transcriptionally active in differentiated muscle cells (44) argues against increased transcriptional activity solely accounting for the greater expression in the 2 week old mice.

The high levels of expression in 2 week old animals were unstable. It occurred in 2 week old ICR and Balb/C mice and with all three promoters (Fig. 5). In older animals, expression was not unstable as we have previously shown (45). In fact, it appeared that total luciferase gravitated to 1 ng/muscle regardless of the initial levels. High levels of luciferase in 2 week old animals decreased to ~1 ng/total muscle and then persisted for at least 22 weeks. Low levels of luciferase also persisted after an initial period of high expression in another study of optimized plasmid constructs in muscle (37). As we had previously shown for older animals (45), luciferase levels rose to ~1 ng/total muscle over the first 8 weeks after injection. Perhaps the immune system is responsible for limiting luciferase expression above the threshold of 1 ng/total muscle. Myofibers expressing high amounts of luciferase could be destroyed by the immune system while myofibers expressing less luciferase escape immune detection. The preliminary studies in which luciferase expression persists in 2 week old animals immunosuppressed by FK506 is consistent with this hypothesis.

The high but transient levels of luciferase expression in 2 week old animals is reminiscent of a similar course following plasmid injections into bupivacaine treated muscle (29). Muscles of both 2 week old mice and mice following bupivacaine treatment contain an increased number of proliferating and fusing myoblasts (46). This state could enable either increased plasmid uptake or increased expression at the transcriptional or translational level as discussed above.

Muscle injections were done in rats, dogs, and non-human primates to determine its possible clinical utility in human infants. For example, newborn screening could identify newborns with Duchenne muscular dystrophy who could be injected as early as 2 weeks of age. It was encouraging that the mice data extended to rats. However, the results in dogs and non-human primates were less promising. It appears that this technique cannot be used to treat intrinsic disorders of muscle, such as Duchenne muscular dystrophy, which require >10% of the muscles to be transfected (47). Nonetheless, the levels of expression in these young animals were substantially greater than in older animals (40). The use of DNA intramuscular injections for immunization may be more effective in infants.

In the laboratory, the ability to obtain such high levels of foreign gene expression in young muscle from pDNA will enable the mouse to be used as cell culture is now used. The transfer of genes into cells in culture has been a critically important tool for deciphering the function of genes. Typically, the gene under study is placed within a plasmid vector and transiently transfected into the appropriate cell in culture. Isoforms and mutant forms of the gene under study can be quickly placed into plasmid expression vectors and studied. The use of pDNA avoids the laborious steps necessary for the production of viral vectors or generation of transgenic mice and thereby enables many different genes and their related mutated forms to be quickly studied. The technique described in this report will permit temporary effects of gene function in muscle to be expeditiously probed within a mammalian organism.

MATERIALS AND METHODS

Animals and injections

C57/Bl, Balb/C and ICR mice, Sprague–Dawley rats and Beagle dogs were purchased from Harlan–Sprague Dawley (Indianapolis, IN). The *mdx-4Cv* dystrophic mice (48) and 129/ReJ-*Lama2*^{dy} mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Rhesus monkeys were bred and maintained at the Wisconsin Regional Primate Center. Animals were anesthetized with metofane (mice), a mixture of Ketamine and Xylazine (rats) and isoflurane (dogs and monkeys). The DNA was injected over a 1 min period using a 1 ml syringe and 27 gauge needle. Muscles were directly exposed to facilitate the injections into specific muscle groups. Incisions were closed using silicone treated silk sutures. All animal protocols were approved by the institutional animal care committee.

Some of the mice received daily intramuscular injections of 0.8 μg/g body weight of FK506 starting 1 day prior to pDNA injection.

Plasmids

Plasmid DNA was purified using QIAGEN plasmid mega kits (Qiagen, Santa Clarita, CA) or by double cesium chloride banding as described previously (40). The construction of pBS.CMVLux, pBS.RSVLux, and pSV40Lux has been described (49). The plasmid pCILuc contains the CMV promoter and a chimeric intron and was constructed by inserting the luc+ gene (*Nhe*I–*Eco*RI fragment) from pSP-luc+ (Promega, Madison, WI) into *Nhe*I and *Eco*RI sites in the multiple cloning site of pCI (Promega). To generate pBS.CMVLuc, the luc+ gene (*Kpn*I–*Eco*RI fragment from pSP-luc+, Promega) was cloned into *Kpn*I and *Eco*RI sites in the multiple cloning site of the pBlueCMV expression vector (49). pCILuc2 was constructed by removing the f1 origin and ampicillin resistance gene from pCR3 (Invitrogen, Carlsbad, CA) by *Stu*I and *Ssp*I digestion followed by religation. The 5′ chimeric intron and multiple cloning site sequences (*Hin*dIII–*Not*I fragment) from pCI (Promega) were inserted into *Hin*dIII–*Bsp*120I sites to replace the original pCR3 multiple cloning site. The luciferase gene (*Nhe*I–*Eco*RI fragment from pSP-luc+) was inserted into *Nhe*I and *Eco*RI sites.

pRSVBecker contains a Becker-like dystrophin gene expressed from a RSV promoter (50). pCIBecker contains a Becker-like dystrophin gene (*Sal*I fragment from pUC18-Becker) inserted into the *Sal*I site of pCI. The pBS.MCKLuc construct contains the mouse muscle creatine kinase gene promoter [3300 bp, (51)] in pBluescript. The luc+ gene and SV40 polyadenylation signal (*Hin*dIII–*Cla*I fragment from pCILuc) were placed downstream of the MCK promoter. pCILux was constructed by inserting the lux gene (*Hin*dIII–*Bam*I fragment pBS.CMVLux) into the *Sma*I site of pCI. Plasmid DNA was fluorescently labeled with rhodamine as described (M. Sebestyen *et al.*, manuscript submitted).

Analyses of foreign gene expression

Luciferase activity in muscle was analyzed as previously reported (21) except muscle extracts were prepared in larger volumes of luciferase lysis buffer (0.1% Triton X-100, 0.1 M potassium phosphate, 1 mM DTT, pH 7.8). The entire muscles of mice and rats were homogenized in 500 μl of lysis buffer using a PRO 200 homogenizer (PRO Scientific Inc., Monroe, CT). The whole muscles of dogs were weighed (0.38–6.7 g) and divided into equal parts of 95–837 mg and then homogenized with 1 ml of lysis buffer. The muscle biopsies from the monkeys (40–180 mg) were homogenized with 500 μl lysis buffer. Supernatants were collected after centrifugation at 4000 r.p.m. at 4°C for 10 min. One to 20 μl of the supernatant were analyzed for luciferase activity. Relative light units (RLU) were converted to pg of luciferase using standards from Analytical Luminescence Laboratories (San Diego, CA). Luciferase protein (pg) = $5.1 \times 10^{-5} \times$ RLU.

Eight μm thick tissue sections were stained for β-galactosidase expression with X-Gal for 8–16 h (21,49). Hematoxylin was used for the counterstain. Dystrophin immunostaining was done using rabbit antibody 6–10 as previously reported (49,50). DNA labeled with rhodamine fluorescence was analyzed on 10 μm sections, fixed in 70% ethanol (4 min) using a Nikon microscope and Photometrics SenSys CCD camera.

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