A PROPIONSAV METABOLIZMUS VELESZÜLETETT RENDELLENESSÉGEI, MOLEKULÁRIS GENETIKAI HÁTTERE ÉS A GÉNTERÁPIA LEHETŐSÉGEI

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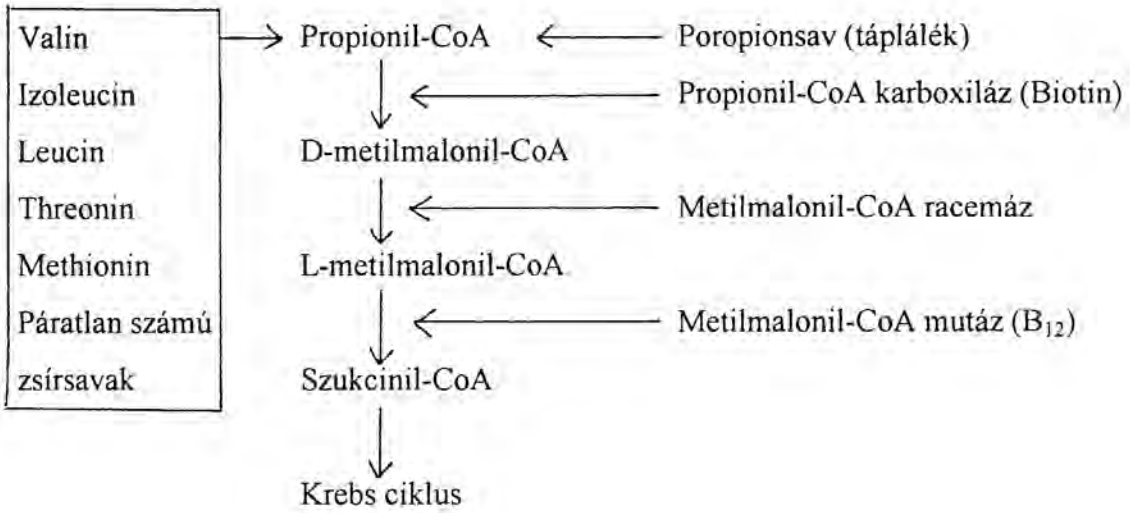
TARTALOM

Irodalmi áttekintés

1. A propionsav metabolizmusa

a.: a metabolizmus fő útvonala:



Az emberi szervezetben a propionsav döntő többségében a különböző elágazó láncú aminosavak és a páratlan szénláncú zsírsavak lebontása során keletkezik (1), valamint kevésbé számottevő mértékben a táplálék emésztése során a béltraktusból szívódik fel (2). A propionsav derivatizációját követően a lebontás fő lépéseit a propionil-CoA karboxiláz, metilmalonil-CoA racemáz és metilmalonil-CoA mutáz enzimek katalizálják, melyet követően a keletkező szukcinil-CoA a Krebs ciklusba lépve metabolizálódik tovább.

b.: a propionsav metabolizmusának alternatív lehetőségei:

Bár az előzőekben említett lebontási útvonal az emlős szervezetekben döntő jelentőségű, létezik a metabolizmus alternatív lehetősége is. Egyik lehetőség, hogy a képződött propionil-CoA a hosszú szénláncú zsírsavak szintézisében (pl.: heptanoát, nonanoát) helyettesítheti az acetil-CoA-t. További lehetőség, hogy részt vesz a telítetlen zsírsavak szintézisében (pl.: acrilil-CoA,), melyek további lebontása során laktát illetve piruvát keletkezik (3,4). Bár a metabolizmus ezen útvonalainak egészséges egyénben alig van jelentősége a propionsav metabolizmus zavarában szenvedő betegekben ezek elsőrendűvé válhatnak (5,6,7).

2. A propionsav anyagcserezavar veleszületett rendellenességei:

a.: propionil-CoA karboxiláz (PCC) deficiencia:

A PCC enzim - mely 6 α és 6 β alegységből álló dodekamer, és működéséhez biotin kofaktor szükséges - genetikai defektusa a "propionsav acidaemia" néven ismert veleszületett anyagcsere-betegségért felelős. A propionsav acidaemiának klasszikusan két formáját ismerjük: ***pccA*** (McKusick 232000), mely az enzim α alegységének, illetve a ***pccB*** (McKusick 232050) mely az enzim β alegységének genetikai defektusából származik. Elkülönítésük fibroblaszt sejtkultúra komplementációs, illetve biotin szenzitivitási vizsgálatokkal lehetséges (8,9,10). Klinikai megjelenésére jellemző az újszülött korban fellépő etetési nehézség, hányás, izomgyengeség, letargia, dehidráció, konvulzió, mely állapot előrehaladtával bekövetkezik a kóma és a kimenetel legtöbbször fatális. Biokémiai vizsgálattal súlyos metabolikus acidózis, ketonaemia, hypoglycaemia, hyperglycinaemia és hyperammonaemia észlelhető. A túlélőknél számolni kell ezen visszatérő jellegű életveszélyes metabolikus krízisekkel, különösen infekciók vagy magas fehérjetartalmú diéta következtében. A kialakuló mentális retardáció az esetek döntő többségében a diétás és gyógyszeres kezelések ellenére is bekövetkezik (11).

b.: metilmalonil-CoA racemáz deficiencia:

Az enzim a D-metilmalonil-CoA - L metilmalonil-CoA átalakulást katalizálja, működéséhez kofaktort nem igényel. Veleszületett hiányáról irodalmi közlés nincs.

c.: metilmalonil-CoA mutáz (MCM) deficiencia:

A veleszületett enzimhiány a "metilmalonsav acidaemia"-ként ismert betegségcsoportot hozza létre. Az apoenzim - mely két azonos alegységből felépülő homodimer - defektusai (McKusick: 251000) két nagy csoportba sorolhatók: **mut^0**, mely az apoenzimet érintő defektusok kétharmadában mutatható ki, diagnózisát az ~~in~~ vitro fibroblaszt tenyészethez excesszív mennyiségben adott kofaktor (kobalamin) jelen~~léte mellett~~ mérhető enzimaktivitás képezi, mely esetben ez kevesebb, mint a normál aktivitás 0.1%-a. Ezen esetek döntő

többségében olyan defektív enzimfehérje szintetizálódik, mely a citoplazmában gyorsan degradálódik.

A **mut**$^-$ jelzésű csoport az apoenzim strukturális abnormalitásából származik, és a kofaktorral szembeni affinitást változtatja meg. Ilyen esetekben a mérhető enzimaktivitás kobalamin hozzáadását követően a normál érték 2-75 %-a között változik (12,13).

A betegség klinikai megjelenésére - különösen a mut^0 csoportban - az újszülött korban fellépő etetési nehézség, letargia, hányás, dehidráció, izomgyengeség, kóma jellemző. A mut$^-$ csoportban előfordulhatnak a későbbi, de még az első életév előtt jelentkező tünetek, melyek közül jellemző a növekedési elmaradás, periódusos hányások, psychomotoros retardáció (14,15). Laboratóriumi vizsgálatokkal súlyos metabolikus acidózis, ketonaemia, hyperammonaemia, gyakran hypoglycaemia, illetve a vérben és vizeletben felgyülemlő nagy mennyiségű metilmalonsav észlelhető (14,16).

Klinikailag és laboratóriumilag hasonló eltérést okozhat a kofaktor - adenozilkobalamin - szintézisének defektusa is (McKusick: 251100, 251110). Ezzel kapcsolatban szintén két fő csoportot különböztetnek meg: **cblA** , melyet a mitokondrialis kobalamin reduktáz defektusa, illetve **cblB** , melyet a kobalamin adenozil transzferáz defektusa okoz. Elkülönítésük szintén szövetkultúrákon végzett enzimvizsgálatokkal lehetséges (17).

d.: multiplex karboxiláz deficiencia:

Ezen kórképet (Mc Kusick: 253270) a biotin metabolizmusának kétféle defektusa hozza létre. Az első csoportra a holokarboxiláz szintetáz hiánya miatt kialakuló, az élet első napjaiban vagy heteiben jelentkező etetési nehézség, izomgyengeség, letargia, görcsök jellemzőek, melyet diffúz bőrpír, alopecia kísérhet (18,19,20,21). A második, egyben gyakoribb csoportra a biotinidáz aktivitás hiánya miatti, inkább a későbbi csecsemő vagy kisdedkorban fellépő széles skálájú neurológiai tünetek (görcs, izomgyengeség, fejlődésbeli elmaradás, halláskárosodás, optikus atrófia) jellemzőek (22,23,24).

A biotin metabolizmus zavara négy biotin függő enzim csökkent működésében nyilvánul meg: acetil-CoA karboxiláz, propionil-CoA karboxiláz, 3-metilkrotonil-CoA karboxiláz, piruvát karboxiláz. A szérum és vizelet vizsgálata ezen enzimhiányok összetett zavarából adódó jellemző metabolit felhalmozódást illetve ürítést mutat. Bár a klinikai kép a propionsav metabolizmusának előzőekben tárgyalt zavaraira emlékeztet, megjelenési formája jóval enyhébb, biotin pótlásra azonnali javulás észlehető és prognózisa sokkal kedvezőbb.

A továbbiakban ezen kórkép részletes tárgyalására nem térek ki.

3. Genetikai áttekintés:

a.: PCC deficiencia:

A PCC deficiencia öröklésmenete autoszomális recesszív, melyet családvizsgálatok igazoltak. Az enzimet felépítő alegységek kromoszómális lokalizációját 1986-ban határozták meg, az ? alegység génje a 13-as kromoszóma q22-q34, míg a ß alegységé a 3-as kromoszóma q13,3-q22 régiójában található. Ugyancsak ebben az évben sikerült az alegységeknek megfelelő humán cDNS-t klónozni (25,26,27,28). A betegekből nyert fibroblaszt kultúrák vizsgálata vezetett el az egyes enzimdefektusokért felelős mutációk azonosításához (29,30). Megjegyeznénk, hogy olyan közlemény, mely *in vitro* körülmények között humán fibroblaszt tenyészetben végzett DNS átviteli technikákat alkalmazva az enzimaktivitás helyreállításáról számolt volna be nem jelent meg.

b.: MCM deficiencia:

Családvizsgálatok alapján az MCM deficiencia ugyancsak autoszomális recesszív öröklésmenetet mutat. A humán MCM gén locusa, melyet 1988-ban térképeztek fel, a 6-os kromoszóma 6p12-21.2 régiójában helyezkedik el (31). A cDNS-t 1989-ben sikerült izolálni (32), a teljes génlocus struktúrájának leírására pedig 1990-ben került sor (33). A betegekből nyert fibroblaszt minták vizsgálatával különböző típusú mutációkat azonosítottak, azonban kiemelkedő frekvenciájú mutációt, vagy a génen speciális, mutációkban gazdag "hot spot" régiót nem találtak (13).

4. A klinikumban jelenleg alkalmazott diagnosztikus és terápiás lehetőségek:

a.: propionil-CoA karboxiláz deficiencia:

A propionsav karboxilációjának zavara felmerül minden újszülöttkori ketózissal vagy acidózissal járó állapotrosszabbodás esetén, miután a gyakori, acidózissal járó kórállapotokat, vagy hasonló megjelenésű veleszületett anyagcsere megbetegedéseket kizártuk. A definitív

diagnózishoz elengedhetetlen a propionsav és metabolitjainak vizsgálata vér vagy vizeletmintából, valamint a propionil-CoA karboxiláz aktivitásának meghatározása leukocita izolátum, vagy fibroblaszt kultúra segítségével. Ez utóbbi tekinthető az egyedüli abszolút specifikus tesztnek, mivel a propionsav felszaporodása a metilmalonsav metabolizmus zavarában is hasonló módon észlelhető. Az enzimvizsgálat nagy rizikójú újszülöttek esetében a köldökvérből izolált leukocita extraktumból is elvégezhető. Prenatális vizsgálatra megbízható eljárásnak tűnik az amniocentézisből nyert sejttenyészetben (34) illetve chorion boholy biopsziátumon (35) végzett [14C]propionsav fixáció mérése, valamint az amnionfolyadék metilcitrát tartalmának meghatározása (36,37).

A kezelést illetően a ketoacidotikus krízisek során a teljes proteinmegvonás, masszív parenterális nártium-bikarbonát pótlás és glükóz infúzió a kezdeti lépés, melyet a hyperammonaemiával járó esetekben peritoneális dialízissel kell kiegészíteni (38). A túlélők számára jelenleg csupán diétás kezelés áll rendelkezésünkre, melynek alapja az alacsony (0,5-1,5 g/kg/die) fehérjebevitel. Bár ezen kezelés mellett a ketoacidotikus krízisek száma minimalizálható, azonban ezek előfordulását teljes mértékben nem akadályozza meg, illetve sok esetben nem teszi lehetővé a normális fejlődést (39). Miután a PCC enzim működéséhez kofaktorként biotin szükséges, ennek nagydózisú adása logikusnak tűnik, ám egyértelmű jótékony hatását bizonyítani nem sikerült (40). Ugyancsak megkérdőjelezhetőek a hosszútávú L-carnitin (mely a kóros metabolitok vizelettel történő ürülését fokozza) és metronidazol (mely a bélflóra által termelt propionsav mennyiségét csökkenti) kezelés hatékonyságáról szóló beszámolók (41,42).

b.: metilmalonil-CoA mutáz deficiencia:

Hasonlóan a PCC deficienciához, az újszülött vagy csecsemőkorban jelentkező ketoacidotikus krízisállapot felveti a metilmalonsav metabolizmusának zavarát. A diagnózis felállításához elégséges a szérum vagy vizeletminta egyszerű kolorimetriás, esetleg gáz kromatográfiás-tömegspektrográfiás vizsgálata. A szérum kobalamin szintjének meghatározása segít a *mut* illetve *Cbl* formák elkülönítésében. Definitív diagnózishoz fibroblaszt tenyészeten végzett, jóval munkaigényesebb vizsgálatok alapján juthatunk, melyek képesek a különböző alcsoportok (mut^0, mut^-, CblA, CblB) pontos elkülönítésére (43). Szintén van lehetőség a prenatális diagnózis felállítására, melyhez egyrészt az amnion folyadék, illetve anyai vizelet metilmalonsav tartalmának meghatározása, másrészt amnion

folyadékból nyert sejttenyészeten, vagy chorion boholy biopsziátumon végzett enzimvizsgálatok szükségesek (36,44,45).

A krízisállapot kezeléséhez a teljes protein megvonás, parenterális glükóz és kobalamin adása, valamint hyperammonaemia esetén peritoneális dialízis szükséges. A krízisek közti időszakban alacsony fehérjetartalmú diéta jelentősen javíthatja még a retardált fejlődésű gyermekek állapotát is (46,47). Az L-carnitinnel és metronidazollal történt próbálkozások eredményei alapján ezek használata nem tekinthető egyértelműnek (41,42).

5. A géntherápia elméleti lehetőségei

A elmúlt évtizedben, a molekuláris genetikában végbement robbanásszerű fejlődés új fejezetet nyitott az orvostudományban a génterápia elméleti és – bár kezdeti stádiumú- gyakorlati alapjának megteremtésével. A génterápia alkalmazása leginkább olyan betegségekben merül fel, melyek kezelése jelenlegi módszereinkkel nem megoldott. E tekintetben a propionsav anyagcsere betegségeiben, melyek még a túlélőkben is igen súlyos következményekkel járnak, a génterápia megfelelő alternatíva lehet. Az eljárás alkalmazásához néhány alapvető kérdést kell megválaszolni: milyen legyen a génátvitel módszere, mi legyen a kiválasztott célszerv, mennyi ideig tartó és milyen szintű génexpresszió mellett számíthatunk javulásra.

A **géntherápia,** elméleti lehetőségét tekintve, két fő csoportra osztható:

a: a tartós **(permanens)** génátvitel, melynek során a kívánt DNS szakasz integrálódik a gazdasejt genomjába, a sejtosztódás során átvívődik az utódsejtbe, és elméletileg folyamatosan expresszálódik. Ilyen megoldás teoretikusan végleges gyógyulást eredményezne.

b: az átmeneti **(tranziens)** génátvitel, mely esetben a bevitt DNS az adott expressziós vektorban marad, a célsejt genomjába nem integrálódik, és rövidebb-hosszabb idő után a sejtből eliminálódik. Ezen módszer alkalmas lehet olyan anyagcsere betegségek kezelésére, melyben a "metabolikus krízisek" periódusosan jelentkeznek, és a köztük eltelt időben a betegek tünetmentesek (IV. sz. közlemény).

Mindkét vonatkozásban történtek kísérletek mind a PCC, mind az MCM deficiencia génterápiás kezelési lehetőségeinek kidolgozására (48,49, I-III sz. csatolt közlemény).

Kísérleti célkitűzések:

1.: PCC deficiencia :

- megkíséreltük a *funkcionális* humán PCC α alegység cDNS-ének klónozását, feltételezve, hogy az irodalomból ismert génszekvencia hibás
- a klónozott cDNS expressziós vektorba történő beültetését és pccA defektusos humán fibroblaszt vonalakba történő bejuttatását követően vizsgáltuk a helyreállított enzimaktivitás mértékét
- vizsgáltuk a propionsav metabolizmus változását géntranszfert követően, a szomatikus génterápia lehetősége szempontjából

2.: MCM deficiencia:

- vizsgáltuk a propionsav metabolizmus változását az MCM enzim cDNS-ét tartalmazó expressziós vektor bejuttatását követően különböző humán sejtvonalakon (mut^0 fibroblaszt, normál fibroblaszt, hepatoma, lymphoma sejtvonalak)
- vizsgáltuk a propionsav metabolizmus változását kevert sejtkultúrás tenyészetekben direkt sejtkontaktus mellett, vagy folyadékfázissal szeparáltan
- ezen kísérletek milyen adatokat szolgáltattak az esetleges "metabolikus kooperáció" kimutatására, és ezen jelenség vonzata a génterápia szempontjából

3.: tranziens génátvitel és génexpresszió *in vivo* egér modellben:

Az asialoglycoprotein/polylysin/DNS komplexről korábban igazolták ennek specifikus, a szisztémás keringésből a májsejt felszíni receptoron keresztül történő felvételét (50,51). Olyan komplexet használva, melynek DNS komponense a metilmalonil-CoA mutáz enzim cDNS-ének expressziós vektorát tartalmazta (ASO/PL/MCM-cDNS), vizsgáltuk ezen komplex szisztémás (egér farokvénán keresztüli) beadását követően:
- a szerv specificitást különböző szövetek analízisével
- a máj által felvett expressziós vektor eliminációját
- az expressziós vektorról átírt mRNS dinamikáját

- az expressziós vektor által létrehozott enzimaktivitást és annak dinamikáját
- az immunreakciót a komplex alkotóelemeivel szemben, ismételt intravénás bevitelt követően

Alkalmazott módszerek:

Ezek részletes ismertetését illetőleg utalok a csatolt közleményekre (I. II. III.)

Eredmények:

A részletes számadatokat illetve ábrákat illetőleg utalok a csatolt közleményekre. Az alábbiak az elért eredmények összefoglalását tartalmazzák.

1. A propioil-Coa karboxilázzal kapcsolatos kísérletek:

a. a PCC enzim α alegységét két részletben sikerült klónozni. A feltételezett szekvenciának megfelelően (GenBank, accession number X14608) tervezett oligonukleotidok segítségével a cDNS 39-970 bp-ig terjedő szakaszához a PCR reakció alapjául humán máj tRNS-t használtunk reverz transzkripciót követően. A 971-2150 bp-ig terjedő szakaszt humán máj fág cDNS könyvtárból sikerült izolálni. Ezen cDNS megfelelt a gén korábban feltételezett transzkripciós iniciációs és stop kodonjának.

b. jelentős különbséget találtunk az általunk klónozott gén és a korábban közölt szekvencia között: ez a 1066-1141 bp-ig terjedő szakaszon 9 nukleotida inzercióját illetve delécióját jelenti, mely e szakaszon 26 aminosav szekvenciájának megváltozását eredményezi. Eredményünket megerősíti, hogy az így kapott aminosav szekvencia csaknem teljes homológiát mutat a patkány PCC α alegységének enzim szekvenciájával.

c. a cDNS 5' végénél lévő mitokondrialis vezető szekvencia rövidebb volt, mint az MCM cDNS hasonló szakasza, az "upstream" szekvenciák klónozására tett erőfeszítéseink nem jártak sikerrel.

d. a kapott klón funkcionális vizsgálataihoz két expressziós vektort szerkesztettünk: I.: a feltételezett átíródó PCCA gén (holoenzim) szekvenciának megfelelő és II.: egy "chimera" gént tartalmazó vektort, mely az MCM gén mitokondrialis vezető szekvenciájából és a PCCA

gén apoenzin régiójából állt. A vektorok promotereként a CMV (cytomegalo virus) korai promoter szerepelt.

e. az expressziós vektorokat elektroporáció segítségével különböző fibroblaszt vonalakba juttatva a [14C]propionsav metabolizmusának változását két módon vizsgáltuk: I.: a fehérjébe történő beépülés, mely a teljes degradációs sorról ad képet, és II.: PCC holoenzim aktivitás. Az incorporációs vizsgálatok bebizonyították, hogy mind az általunk klónozott PCCA gén, mind a chimera gén a pccA fibroblasztokban a fehérjébe történő beépülést normál szintre korrigálta, nem változott a mért érték normál, pccB és mut^0 sejtekbe történő bejuttatáskor, a DNS mennyiség növelésével a metabolikus aktivitásban további emelkedést elérni nem lehetett.

f. az enzimaktivitás (melyet a propionát-szukcinát átalakulás mérésével végeztünk) a pccA sejtkultúrában a normál érték csupán 10-20%-a volt, mely a bevitt DNS mennyiségének növelésével sem változott, ugyancsak nem lehetett az enzimaktivitás növekedését észlelni normál sejtekben.sát.

Következtetések:

i, ez az érték megfelelt az elektroporáció hatékonyságának (azaz a tenyészet sejtjeinek kb. 10-20%-ában került felvételre a gén), ezt párhuzamos kísérletekben β-galaktozidáz (β-gal) enzim génjét tartalmazó expressziós vektor elektroporációjával ellenőriztük, melynek lényege, hogy azon sejtek, melyekben a gén expresszálódik, megfesthetők

ii, az α alegység overexpressziója (bevitt DNS mennyiségének növelése) ellenére a PCC gén β alegységének overexpressziójára - magától értetődően - nem került sor, így az α alegység "overexpresszió nem eredményezhet többletenzim képződést

iii, mivel így is sikerült a teljes sejtkultúrára nézve normál metabolizmust helyreállítani, feltehetően léteznie kell a sejtek között "metabolikus kooperáció"-nak.

2. A metilmalonil-CoA mutázzal kapcsolatos kísérletek:

A propionsav metabolizmus változását vizsgáltuk különböző sejtkultúrákban MCM cDNS-t tartalmazó expressziós vektor elektroporációval történő bejuttatását követően. A kísérletekben a már korábban leírt pCMV-MCM expressziós vektort alkalmaztuk.

a. az MCM enzimaktivitás változását mértük normál, *mut*^{0}és hepatoma sejtvonalakon. Az expressziós vektor bejuttatását követően az enzimaktivitás (propionát-szukcinát átalakulás) értéke a normál sejtekben ötszörös, *mut*^{0} (MCM deficiens) sejtekben a normál sejtek alapaktivitását meghaladó, illetve hepatoma sejtekben az alapaktivitás kétszeres növekedést mutatott

b. már alacsony (10-20% normál) enzimaktivitásnál is a propionsav metabolizmusa normális értéket ért el

c. az enzimaktivitás növekedése dózisfüggőnek bizonyult, a bevitt DNS mennyiségével párhuzamosan, szemben a PCC α alegységével végzett kísérletekkel

d. a teljes propionsav metabolizmus változását vizsgálva (propionát-fehérje beépülés) a fenti körülmények között azt tapasztaltuk, hogy míg a *mut*^{0} sejtekben a normál metabolizmus helyreállt, addig normál sejtekben, illetve hepatoma sejtekben az inkorporáció mértéke nem változott

Következtetések:

i, ellentétben a PCC génnel, (mely heterododekamer) az MCM gén (homodimer) overexpressziója funkcionális enzimfehérje többletszintézisét hozza létre, mely az enzimaktivitás DNS-dózis függő emelkedését eredményezi

ii, hasonlóan a PCC kísérletekhez már alacsony (10-20% normál) enzimaktivitásnál is a totál propionsav metabolizmus normális értéket ért el *mut*^{0} sejtekben, melyből szintén következtetni lehet a sejtek közti "metabolikus kooperáció" jelenlétére

iii, az MCM enzimaktivitás tekintetében normál sejtekben (normál fibroblaszt, hepatoma sejtvonal) az enzim overexpressziója a propionsav metabolizmusát (propionát-fehérje) nem növeli, azaz a teljes metabolikus sor sebességének nem ez a meghatározó („rate limiting step") eleme

3. Kevert sejtkultúrák a "metabolikus kooperáció" igazolására:

Hogy feltételezésünket bizonyítsuk, a géntranszferes kísérletek összes lehetséges buktatóját ki kellett küszöbölni. A jelenség modellezésére a különféle sejtek stabil, meghatározott arányú kevert tenyészeteiben történő mérések szolgáltak.

a. diszproporcionálisan magasabb volt a propionsav fehérjébe történő beépülése, ha normál sejteket kevertünk 10% illetve 30 % arányban *mut*^{0} sejtekhez, ekkor a normál metabolikus aktivitás 30%-át illetve 70%-át tudtuk mérni

b. ugyancsak megemelkedett a propionát beépülés (mintegy a normál 40-50% -ára), ha *pccA* és *mut*^{0} sejteket kevertünk össze, holott ezek külön-külön jelentős beépítésre nem képesek

c. aktivitásnövekedést detektálni egymástól membránnal elválasztott sejtek esetében nem tudtunk

Következtetések:

i., létezik a sejtek között metabolikus kooperáció

ii., ehhez kísérleteink alapján sejt-sejt kontaktusra van szükség

iii., génterápia során nem kell valamennyi defektusos sejtben az enzimaktivitást helyreállítani ahhoz, hogy normál metabolizmust érjünk el

4. *In vivo* génátvitel ASO/PL/DNS(MCM-CMV expressziós vektor) komplexxel:

Az ASO/PL/DNS komplexet, melynek DNS tartalmát a sejtkultúrás kísérletekben is használt MCM-cDNS CMV expressziós vektor jelentette, egér farokvénájába fecskendeztük, az antigén képződéssel kapcsolatos kísérletekben több alkalommal, mintegy 8 hónapos periódus alatt.

a. a beadást követően szérumból sorozat-meghatározások alapján a komplex $t_{1/2}$=2.5 min féléletidővel eliminálódott, 30 percen túl kimutatni nem lehetett

b. a beadás után egy órával vizsgáltuk a szöveti disztribúciót, a felvett DNS a legmagasabb koncentrációt a májban érte el, sokkal kisebb mértékben kimutatható volt még lépből, tüdőből, alig detektálható volt egyéb szövetekben

c. a komplex eliminációját vizsgálva a DNS mintegy 24 óra múlva már csak nyomokban volt kimutatható a májban, féléletidő $t_{1/2}$=1-1.3 óra volt.

d. ez a nyomokban kimutatható mennyiség még 30 nap után is detektálható volt, metilációs vizsgálatok alapján ez a DNS a komplexből származott, a genomba történő integrálódást kimutatni nem lehetett.

e. májszövetből RT-PCR alkalmazásával vizsgálva az expressziós vektorból a 6-24 óra időtartam között transzkripciót tudtunk igazolni

f. ugyancsak májszövetből vizsgálva, a beadást követő 24-48 óra időtartamban az enzimaktivitás az alapaktivitás fölé emelkedett mintegy 30-40%-kal

az antigenitási kísérletek eredménye:

g. akut reakció első beadást követően egy alkalommal sem volt

h. négy, többször injektált egérből egy a negyedik beadást követően 10 percen belül kimúlt, feltehetően anaphylaxiás reakciót követően

i. a túlélő egerekben, ezeket 8 hónap után feláldozva, rutin szövettani vizsgálatokkal krónikus toxicitásra utaló jeleket nem észleltünk

j. a keringő ellenanyagok vizsgálatához [125I]-jelzett komplexet használva, ezzel mindhárom állat széruma reagált

k. komplementációs vizsgálatokkal igazoltuk, hogy az ellenanyagok a komplex ASO illetve PL komponensei ellen képződtek, a DNS ellen képződött antitest jelenlétét kimutatni nem tudtunk

l. a klinikai gyakorlatban használatos antinukleáris antitest (ANA) reakció is minden esetben negatív volt

A kísérleti eredményekből levonható következtetések, és a gyakorlati alkalmazás lehetőségei:

1. Sikeres volt a propionil-CoA karboxiláz enzim á alegység cDNS-ének klónozása, melyről más fajjal összehasonlítva kimutattuk a szoros homológiát és konzervativizmust.
2. Igazoltuk ezen gén funkcionális aktivitását pccA hiányos humán fibroblaszt tenyészetben, mely diagnosztikus céllal is alkalmazható.
3. Mind a propionil-CoA karboxiláz, mind a metilmalonil-CoA mutáz enzim génjét tartalmazó expressziós vektorral igazoltuk a propionsav metabolizmus helyreállítását defektusos sejtvonalakon.
4. A sejtekbe juttatott DNS dózis-metabolizmus összefüggés vizsgálattal felvetettük a sejtek közötti metabolikus kooperáció lehetőségét.
5. Kevert sejtkultúrás kísérletekkel igazoltuk a fenti jelenség létezését: kimutattuk, hogy ehhez sejt-sejt direkt kapcsolatra van szükség, ebből a génterápia tervezését illetően (célszövet, transzfekciós hatásfok, expresszió mértéke) vonhatók le következtetések.
6. *In vivo* egér modellen szisztémás bevitelt követően kimutattuk az aszialoorosomukoid/polilizin/MCM-cDNS komplex alkalmazhatóságát: demonstráltuk az expressziós vektor specifikus felvételét májszövetben, leírtuk a kapott génexpresszió, transzláció, elimináció dinamikáját.
7. Vizsgáltuk a komplex elleni immunreakciót: kimutattuk, hogy az ellenanyag képződés a komplex fehérje komponensei ellen irányul, a bejuttatott DNS komponens ellenanyag reakciót nem vált ki.
8. Az alkalmazott molekuláris genetikai módszereket sikerült bevezetni a POTE Orvosi Genetikai Intézet Laboratóriumában.
9. Ennek köszönhetően ezen módszerekkel tisztázható a propionsav metabolizmus zavarában szenvedő betegek diagnózisa. Ez jelentős előrelépés a veleszületett anyagcsere betegségek kivizsgálásában.
10. Bár az értekezés alapjául szolgáló közleményekben tárgyalt kísérletek külföldön kerültek elvégzésre, a módszerek pécsi alkalmazása lehetővé tette hazai molekuláris genetikai diagnosztikus és epidemiológiai vizsgálatok kivitelezését (52-60).

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Cloning of Functional Alpha Propionyl CoA Carboxylase and Correction of Enzyme Deficiency in *pccA* Fibroblasts

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Summary

Propionyl CoA carboxylase (PPC) is a heteromeric enzyme composed of α subunits (PCCA) and β (PCCB) subunits. We describe cDNA clones expressing human PCCA and complementation of the genetic defect in *pccA* fibroblasts by DNA-mediated gene transfer. Two cDNA clones were constructed. The first corresponds to the previously reported, putatively full-length, open reading frame. The second encodes a chimera composed of the mitochondrial leader sequence of human methylmalonyl CoA mutase and the mature PCCA protein. Both clones reconstitute propionate flux to normal levels in fibroblasts from patients genetically deficient in PCCA (*pccA*). The maximal level of propionate flux approached, but never exceeded, the levels seen in control plates of normal cells. In contrast, the maximal level of PPC holoenzyme activity reached only 10%–20% that of normal controls, which corresponded roughly to the fraction of cells actually transformed with the recombinant gene. These data suggest that the level of PCCA expression in fibroblasts does not normally limit PCC holoenzyme activity or propionate flux. The fact that a small fraction of cells reconstitutes propionate flux to normal levels suggests that metabolic cooperation between cells is capable of increasing the metabolic capacity of recombinant enzyme in a subpopulation of cells. These factors may have important implications for the rational design of somatic gene therapy for PCCA deficiency.

Introduction

Metabolism of propionate in higher eukaryotes proceeds by derivatization of this volatile fatty acid to propionyl CoA, followed by degradation via the enzymes propionyl CoA carboxylase (PCC; E.C.6.4.1.3), methylmalonyl CoA racemase, and methylmalonyl CoA mutase (MCM; E.C.5.4.99.2). PCC is a heteromeric dodecamer composed of α subunits (PCCA) and β (PCCB) subunits. Genetic deficiency of PCC causes an inborn error of metabolism, termed "propionic acidemia." Two distinct genotypic forms of propionic acidemia are classically distinguished by somatic cell complementation: *pccA* (McKusick 23200), resulting from inherited defects in the α (PCCA) gene, and *pccBC* (McKusick 23205), resulting from inherited defects in the β (PCCB) gene. PCCA and PCCB cDNA clones have been reported elsewhere (Kraus et al. 1986; Lamhonwah et al. 1986), and mutations in the PCCA gene have been identified which are associated with *pccA* forms of propionic acidemia (Ohura et al. 1989).

Both *pccA* deficiency and *pccBC* deficiency cause profound disturbances in intermediary metabolism. Infants can present with severe metabolic acidosis, hyperglycinemia, and hyperammonemia in the newborn period, which is often fatal. Survivors often have recurrent episodes of life-threatening acidemia and mental retardation, despite dietary and pharmacological management (Rosenberg and Fenton 1989).

Our laboratory is interested in the molecular and biochemical processes which will be involved in somatic gene therapy for organic acid disorders. In studies directed at developing somatic gene therapy for MCM deficiency (methylmalonic acidemia; McKusick 251000), we have demonstrated the complementation of MCM deficiency in fibroblasts from patients

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with *mut* forms of methylmalonic acidemia, by DNA-mediated gene transfer (Wilkemeyer et al. 1991, and in press; Andrews et al., submitted), as well as permanent restoration of MCM activity in fibroblasts transduced with a recombinant retroviral vector carrying the human MCM gene (Sawada and Ledley, in press). These studies have also addressed the metabolic consequences of overexpressing recombinant MCM, the constraints on metabolic flux which will determine the optimal somatic target for gene therapy, and the level of recombinant gene expression required to obtain a biological effect (Wilkemeyer et al., in press).

The present studies are directed at considering somatic gene therapy for propionic acidemia. We describe the cloning of a functional PCCA cDNA clone, demonstrate that DNA gene transfer of this clone will complement the genetic defect in *pccA* fibroblasts, and provide an initial assessment of the metabolic interactions which will be involved in constituting propionate flux for the purposes of somatic gene therapy.

Material and Methods

Amplification and Cloning of the Open Reading Frame of PCCA

Oligonucleotides corresponding to the published sequences of the proposed translation initiation and termination codons of human PCCA (GenBank accession number X14608) were synthesized (Lamhonwah et al. 1986). Additional oligonucleotides were synthesized to internal sequences. Oligonucleotide sequences were modified to include restriction-endonuclease sites, without altering the reported amino acid sequence (fig. 1).

Total RNA was reverse-transcribed using random oligonucleotide primers, and this material was amplified by PCR as described elsewhere (Innis et al. 1990). PCR was performed on phage constituting a human liver cDNA library (Kwok et al. 1985), as described elsewhere (Jansen and Ledley 1990). PCR conditions included 30 cycles at 92°C for 1 min, 61°C for 1 min, and 74°C for 1.5 min. PCR products were subcloned after restriction-enzyme digestion into pGEM-3Zf (Promega). The complete open reading frame was reconstituted by three-part ligation (fig. 1, *top*).

The human MCM cDNA sequence has been reported elsewhere (Jansen et al. 1989). A chimeric clone encoding the mitochondrial leader sequence of human MCM (Jansen et al. 1989) and the mature PCCA protein was constructed (fig. 1, *bottom*). PCR was performed to amplify sequences encoding the mitochondrial leader sequence from human MCM, and a *Dra*I (blunt) restriction site was inserted at the 3′ end of these sequences. Similarly, sequences encoding the mature PCCA protein were amplified by PCR, and a *Sma*I (blunt) restriction site was inserted at the 5′ end of these sequences. These two fragments were recombined in pGEM-3Zf plasmid by three-part ligation (fig. 1, *bottom*). Three independent clones were se-

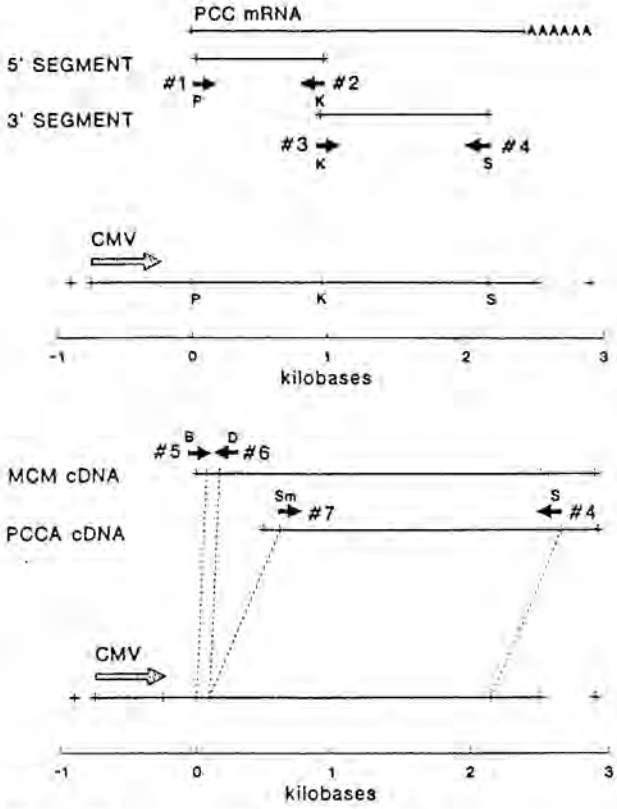

Figure 1 Cloning of human PCCA and expression vectors. *Top,* Bases 39–970 of PCCA, amplified by PCR after reverse transcription of total liver RNA, and bases 971–2157, amplified from a human liver cDNA library. These segments were recombined by three-part ligation and were cloned into the expression vector pNAss-CMV. *Bottom,* Chimeric gene constructed from the mitochondrial leader sequence of the human MCM cDNA and from the region coding the mature (processed) protein of PCCA. Segments were amplified from the respective cDNA clones by PCR and were recombined by three-part ligation into the expression vector pNAss-CMV. P = *Pst*I; K = *Kpn*I; S = *Sal*I; B = *Bam*HI; D = *Dra*I; Sm = *Sma*I; and CMV = cytomegalovirus immediate early promoter. Oligonucleotide sequences are as follows: 1, GCGCTGCAGCTGATGCTGAGC; 2, CAAGGAACTCCACGGTACCAGCAG; 3, ATATTCCTCTGCTGGTACCGTGG; 4, TATGTCGACTTCATTCCAGCTCCACGAGCA; 5, GTGTTTGGATCCTCCACCATGTTAAGAGCT; 6, CTGGTTTAAAAGTCGTTGCTGTATGAGCCT; and 7, CTGAACCCGGGTTCAGTGGGATATGATCC.

quenced in both directions by using double-stranded, dideoxy sequencing (USB Sequenase version 2.0 kit).

Gene Transfer and Expression of PCCA

Sequences constituting the open reading frame of PCCA or the chimeric MCM/PCCA construct were subcloned into the vector pNAss-CMV (MacGregor and Caskey 1989) by blunt-end ligation (fig. 1). Primary *pccA* and *pccBC* fibroblasts were provided by Dr. Wayne Fenton (Yale University). *Mut* and normal fibroblasts have been described elsewhere (Ledley et al. 1990). Expression-vector plasmids were introduced into primary fibroblasts by electroporation using a BioRad Genepulser™ according to a method described elsewhere (Wilkemeyer et al. 1991). Electroporation was performed with 5×10^5 cells in 300 μl, with 20 μg of DNA (unless varied as indicated), 260 V, and 960 μF. Cells were plated at 1.5×10^5 cells/12-mm round culture chamber (12-well plate). Control experiments with β-galactosidase were performed periodically to ensure consistency of electroporation conditions.

Incorporation of [^{14}C]-propionate into trichloroacetic acid–precipitable material was measured according to a method described elsewhere (Ledley et al. 1990), 48 h after electroporation, during the transient phase of gene expression. PCC holoenzyme activity was measured in protein extracts harvested 48 h after electroporation, by using the method of Gravel et al. (1977), which measures the fixation of [^{14}C]-HCO_3 and incorporation into acid-precipitable material. Assays were performed on 25 μg of protein harvested from trypsinized cells by three freeze-thaw cycles (−70°C and 20°C) in 0.05 M Tris-HCl (pH 8), 0.025 M KCl. Protein concentration was determined by Bio-Rad assay. For cocultivation, cells were plated on 12-well (12-mm) plates at a density of 150,000 cells/well after overnight treatment with mitomycin-C at a concentration of 5 μg/10^6 cells.

Results

Cloning and Sequencing the Open Reading Frame of Human PCCA

Sequences corresponding to the reported open reading frame of PCCA were amplified by PCR, subcloned, and sequenced. The 5′ end of the cDNA from base 39 to base 970 was cloned from reverse-transcribed human liver RNA, and the 3′ end of the cDNA from base 971 to base 2157 was cloned from a human liver cDNA library (Kwok et al. 1985). A substantial discrepancy was identified between the sequence of our clones and that reported elsewhere (Lamhonwah et al. 1986), involving nine missing or inserted nucleotides between base 1066 and base 1141 (fig. 2). These changes alter the predicted reading frame comprising 26 amino acids between amino acid 339 and amino acid 365. The reading frame predicted from our sequence preserves apparent homology to the rat PCCA sequence reported by Browner at al. (1989) (fig. 2).

Reconstitution of Propionate Incorporation in PCCA-deficient Cells with PCCA or MCM/PCCA cDNA Clones

Sequences corresponding to either the putative reading frame of PCCA or the chimeric MCM-PCCA construct were recombined in an expression vector which utilizes the CMV immediate early promoter (fig. 3) and were electroporated into PCCA-deficient cells as well as into control cells including normal, *pccBC*, and *mut* fibroblasts. Incorporation of [^{14}C]-propionate into TCA-precipitable material is dependent on the activity of both PCC and MCM, as evidenced by the

```
      1064 1070      1080      1090      1100      1110      1120      1130      1140      1150
REPORTED HUMAN PCCA SEQUENCE
AA    IleHisTrpProGlyProSerProGlyLys Thr ValLeuGln  GluHis Leu SerGlyThrAsnLysLeuIlePheAlaPheAsn
DNA   ATTCACTGGCCTGGACCTAGTCCAGGAAA*GA*CCGTGTTGC*A*GGAGC*ACC*TCTCAGGCACAAACAAGCTGATATTCGCATTCAACGG...
DNA   ATT*ACTGGCCTGGACCTAGTCCAGGAAATGATCCGTGTTGCTAAGG*GCTACCCTCTCAGGCACAAACAAGCTGATATTCGCAT*CAACGG...
AA    Ile ThrGlyLeuAspLeuValGlnGluMetIleArgValAlaLysGly TyrProLeuArgHisLysGlnAlaAspIleArgIle Asn...
REVISED HUMAN PCCA SEQUENCE

AA    Ile ThrGlyLeuAspLeuValGlnGluMetIleLeuValAlaLysGly TyrProLeuArgLisLysGlnGluAspIleProIle Ser
DNA   ATT*ACTGGCCTGGACTTAGTCCAAGAAATGATCCTGGTTGCTAAGG*GTTACCCACTCAGGCACAAGCAAGAGGATATTCCCAT*CAGTGG...
RAT PCCA SEQUENCE
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Figure 2 Revised sequence of human PCCA. Differences between the PCCA sequence published by Lamhonwah et al. (1986) and the sequence determined in these studies are shown. The revised protein sequence (*underlined*) has a different reading frame and preserves identity to the rat PCCA protein sequence reported by Browner et al. (1989) and revised by W. Fenton and L. E. Rosenberg (personal communication).

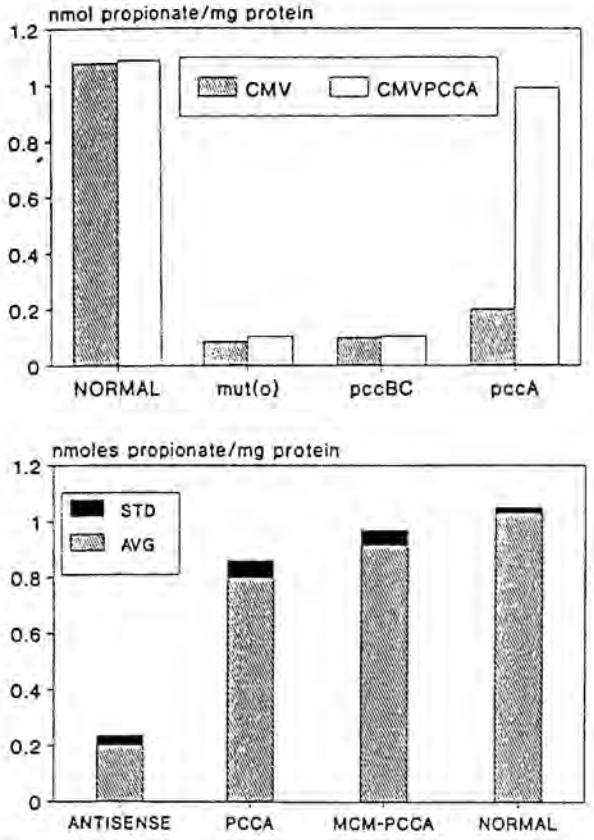

Figure 3 Complementation of *pccA* deficiency by DNA-mediated gene transfer of PCCA and chimeric MCM-PCCA cDNA clones. Expression vectors containing PCCA or a chimeric MCM/PCCA cDNA clone under transcriptional control of a CMV promoter were introduced into mutant fibroblasts by electroporation, and propionate incorporation was assayed. *Top*, Electroporation of PCCA into normal fibroblasts, *mut*^{0} fibroblasts (MCM deficient), *pccBC* fibroblasts (PCCB deficient), and *pccA* fibroblasts (PCCA deficient). *Bottom*, Electroporation of PCCA and chimeric MCM/PCCA clones into *pccA* fibroblasts. Controls include electroporations using antisense PCCA and assays of propionate uptake in normal fibroblasts.

lack of significant [^{14}C]-propionate incorporation in cells genetically defective in these enzymes. [^{14}C]-propionate incorporation was restored essentially to normal levels when either the PCCA clone (fig. 3, *top*) or the chimeric MCM-PCCA clone (fig. 3, *bottom*) was introduced into *pccA* cells (fig. 3, *top*). Introduction of the PCCA cDNA or chimeric MCM/PCCA clone into *pccBC* cells or *mut* cells did not increase propionate incorporation.

Several aspects of these data are of particular significance. First, the maximal level of reconstituted propionate incorporation approached the level in normal cell cultures, even though control experiments demonstrate that the conditions used for electroporation consistently result in expression of a β-gal marker gene in 10%–20% of cells. Second, gene transfer of the PCCA cDNA or chimeric MCM/PCCA clone into cultures of normal cells did not increase the level of propionate incorporation above normal (fig. 3).

Constitution of PCC Holoenzyme Activity in Transfected Cells

PCC holoenzyme activity was measured in protein extracts from transfected *pccA* cells (PCC427) (fig. 4). Electroporation with the empty CMV vector had no effect on enzyme activity. Low levels of PCC activity were apparent in PCC427 transfected with either the PCCA clone or the chimeric MCM-PCCA clone (fig. 4). Significantly, the level of PCC holoenzyme activity, calculated as a fraction of normal activity (10%–20%), is comparable to the fraction of cells which express a marker gene (β-galactosidase) in control experiments performed using identical electroporation conditions and vectors. There was no increase in propionate incorporation or assayable PCC activity when 0.1 μg Biotin/ml was added to the culture media (data not shown). Thus, the level of enzyme activity in the subpopulation of cells which express the recombinant gene is close to the level in normal fibroblasts. No statistically significant increase in PCC activity was observed with identical electroporation into normal cells.

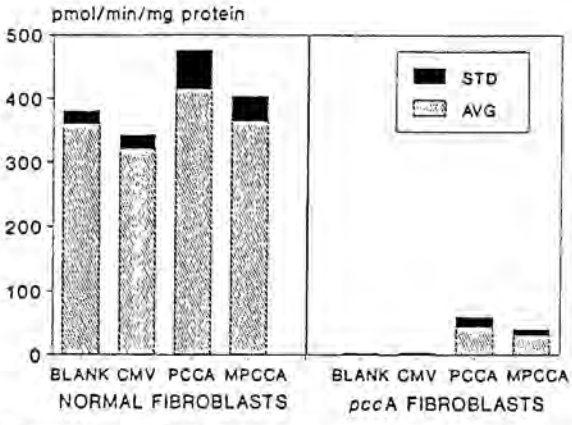

Figure 4 PCC holoenzyme activity in fibroblasts after DNA-mediated gene transfer of PCCA or chimeric MCM/PCCA clones. PCC holoenzyme activity was measured in protein extracts of normal fibroblasts or *pccA*-deficient fibroblasts after electroporation of the CMV vector alone (CMV), vector containing the PCCA cDNA (PCCA), or the MCM/PCCA chimeric cDNA (MPCCA), as well as in cells not exposed to electroporation.

Dynamics of Propionate Metabolism in Transfected Cells

To further investigate the level of PCC activity required to reconstitute propionate flux, propionate incorporation was measured in response to electroporation with different amounts of the vector DNA (fig. 5, *top*). In these experiments the conditions for electroporation were identical in each reaction, with the total amount of DNA maintained at 20 μg by diluting the PCCA vector with the empty CMV vector. Significant levels of [^{14}C]-propionate incorporation are observed with < 1–2 μg of DNA. Moreover, propionate incorporation was nonlinearly related to DNA concentration, with levels approaching, but never exceeding, the level in normal fibroblasts.

PCC holoenzyme was assayed in protein extracts from cells electroporated under identical conditions. PCCA activity paralleled the level of propionate flux, approaching levels which were 20% of the level in normal cell cultures. As in previous experiments, this fraction was similar to the fraction of cells transformed in control experiments with the β-galactosidase marker gene. In control experiments (not shown), we have demonstrated that the level of expression of another gene (MCM) continues to increase proportionally to the amount of DNA added in a range of 1–60 μg of DNA.

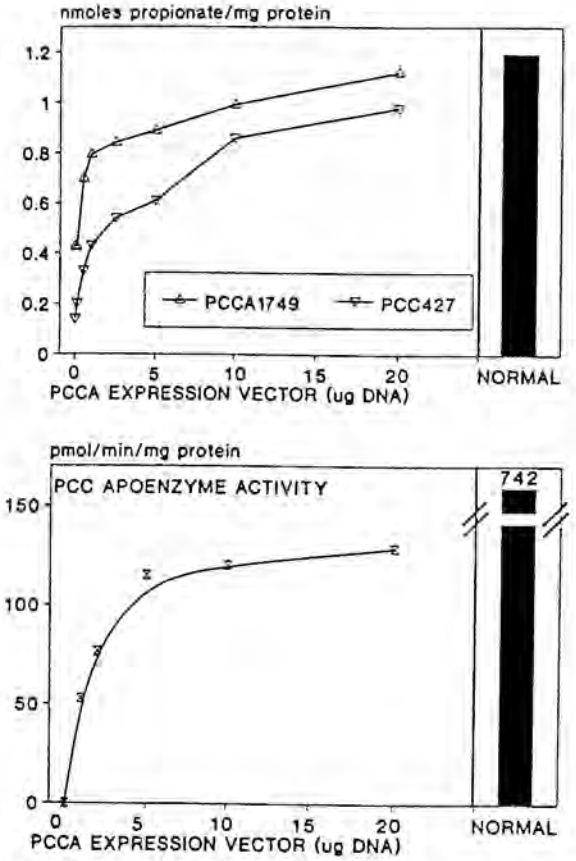

Figure 5 Relationship between amount of DNA in electroporation and complementation of propionate incorporation and PCC holoenzyme activity. *Top*, Propionate incorporation in two *pccA* cell lines (PCCA1749 and PCCA427) electroporated with varying amounts of the PCCA expression vector. The total amount of DNA was maintained at a constant level by addition of the empty CMV vector. Maximal activity in both cell lines approached but did not exceed the levels observed in normal control fibroblasts. *Bottom*, PCC holoenzyme activity in PCCA1749 cells electroporated under identical conditions. The increase of activity parallels that observed for propionate incorporation, but maximal levels are only 20% that in normal controls. This fraction resembles the fraction of cells which express the β-galactosidase marker gene in the same CMV vector after electroporation using identical conditions.

Metabolic Cooperation between Normal and PCCA Cells

To assess whether the disproportionately greater reconstitution of propionate flux relative to holoenzyme activity was an artifact of exposing cells to electroporation, [^{14}C]-propionate incorporation was measured in cocultures containing different dilutions of normal fibroblasts and *pccA* fibroblasts (fig. 6). These results demonstrate that propionate incorporation in mixed cultures is disproportionately greater than the fraction of normal cells in the culture.

Discussion

Inherited deficiencies of PCC are associated with significant mortality and morbidity, despite conven-

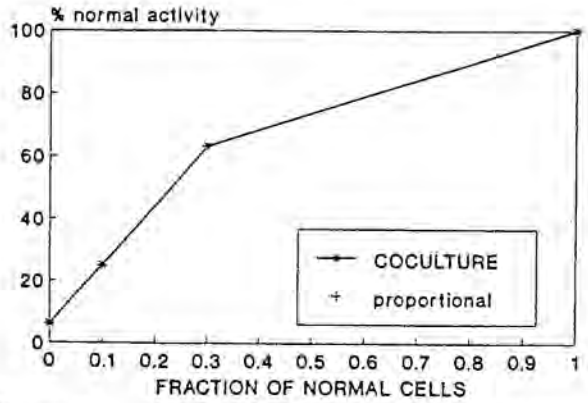

Figure 6 Propionate incorporation in cocultures of normal and *pccA* fibroblasts. Propionate incorporation in cocultures containing different ratios of normal and *pccA* fibroblast. The level of propionate incorporation was observed to be disproportionately greater than the fraction of normal cells.

tional therapy, and may be appropriate disorders for somatic gene therapy. Both the PCCA subunit and the PCCB subunit of this multimeric enzyme have been cloned previously (Kraus et al. 1986; Lamhonwah et al. 1986), but these studies have not demonstrated enzymatic activity of the putative full-length cDNA clones. The present work describes (*a*) cloning and sequencing the putative open reading frame of the α subunit of the human PCC gene and (*b*) complementation of the defect in PCC enzyme activity and propionate metabolism in *pccA* fibroblasts by DNA-mediated gene transfer. The ability to complement the defect in *pccA* cells by gene transfer of these clones confirms the association of the *pccA* defect with this genetic locus (Hsia et al. 1971; Gravel et al. 1977; Wolf et al. 1978, 1980; Lamhonwah et al. 1983; Lamhonwah and Gravel 1987) and suggests that in the future it may be feasible to consider somatic gene therapy for this deficiency state.

One of the problems encountered in this work was the uncertainty whether the published human PCCA sequence was complete and whether the reported reading frame was entirely correct. Significant differences were identified in the open reading frame of our clones and in the sequences reported previously. Moreover, there is some uncertainty whether the reported human PCCA sequence contains the complete mitochondrial leader sequence. The reported cDNA clone has an AUG and putative mitochondrial leader sequence upstream from the determined amino-terminal end of the mature protein. The rat mitochondrial leader sequence (provided by Dr. Wayne Fenton) contains an additional 16 amino acids 5′ to the human AUG. When the putative 5′ untranslated sequences from the human cDNA are translated, there is evident homology with the rat mitochondrial leader sequence (not shown), suggesting that there is evolutionary pressure for preservation of these sequences. Primer extension data from our laboratory (data not shown) suggest that there are ≥50 bases 5′ to the putative AUG of the human open reading frame, although thus far we have not been able to clone these sequences. Because of our concern about the completeness of the PCCA mitochondrial leader sequence, we constructed clones corresponding to both the reported human sequence and a chimera with sequences encoding the mitochondrial leader sequence from human MCM and the mature PCCA protein. Significantly, both the chimeric gene containing the MCM leader sequence and the gene containing the reported open reading frame of PCCA expressed biological activity. Thus, there is sufficient information in the described mitochondrial leader sequence to direct assembly of a functional PCCA subunit even if this sequence is incomplete.

The ability to complement the defect in *pccA* cells by gene transfer may provide means for differential diagnosis of *pccA* deficiency vis-à-vis *pccBC* deficiency. This method for diagnosis would be important for predicting the susceptibility of individual patients to gene-replacement therapy (Wilkemeyer et al. 1991).

The reconstitution of propionate metabolism in *pccA* cells after gene transfer exhibited several quantitatively important characteristics. The first observation is that constituting low levels of assayable enzyme activity restored propionate flux to essentially normal levels. This would provide an explanation for the clinical observation that mutations which leave little residual activity may cause a less severe clinical phenotype (Wolf et al. 1979).

The second observation is that the maximal level of PCC holoenzyme activity never exceeds 10%–20% of normal cultures. This fraction correlates with the efficiency of electroporation—namely, the number of cells which stain with X gal after control electroporation with identical constructs containing the β-galactosidase reporter gene. In addition, no consistent increase in holoenzyme activity was observed after electroporation of normal cells. In light of the fact that PCC is a heteromeric protein, the failure to overexpress PCC holoenzyme activity could be explained by limitations in the amount of the β subunit in each cell under conditions used in these experiments. This hypothesis differs from the predictions of Ohura et al. (1989), which suggest that the β subunit is normally synthesized in excess and is rapidly degraded unless complexed with α subunit. In the future, both hypotheses can be tested with expression vectors for PCCB.

The third observation is that, with increasing amounts of DNA, the maximal level of [^{14}C]-propionate incorporation in transfected cultures approached, but never exceeded, the levels in normal cell cultures. Again, this level of propionate flux was achieved with conditions which introduce the recombinant gene into only 10%–20% of cells. A disproportionately high level of propionate incorporation relative to the fraction of metabolically competent cells was also observed in cocultures of PCCA and normal cells, suggesting that this was not simply an artifact of electroporation.

A similar disproportionality between propionate flux and the fraction of transfected cells has been observed after genetic complementation of MCM deficiency (Wilkemeyer et al., in press). These studies sug-

gested that there was a rate-limiting reaction in propionate flux, which could not be increased by overexpression of MCM. These studies also suggested that metabolic cooperation between adjacent cells enabled the products of the limiting reaction to move between cells, thus increasing the flux through MCM in the subpopulation of genetically reconstituted cells. The potential for metabolic cooperation was demonstrated by cocultivation of *pcc* and *mut*. These cocultures exhibit higher levels of propionate metabolism than does either mutant cell line alone, suggesting that the products of the PCC reaction can move between cells and serve as substrates for MCM (Wilkemeyer et al., in press). In typical experiments the level of [^{14}C]-propionate incorporation, which is 1.8–2.5 nmol propionate/mg protein in *mut* or *pcc* cells, will increase to 4–5 nmol propionate/mg protein in cultures containing 1:2 or 2:1 mixtures of these two cell lines. In contrast, when one cell line is grown on a Transwell™ membrane and the other is grown in the same well below this membrane, there is no increase in propionate incorporation (Wilkemeyer et al., in press).

The saturation of propionate incorporation in *pccA* cells transfected with PCCA provides more evidence for the presence of a rate-limiting reaction in this pathway. We believe that the rate-limiting process involves a step in the provision of the acyl-CoA substrate for PCC. However, since we were unable to overexpress PCC holoenzyme activity, the present experiments do not exclude the theoretical possibility that PCC itself may be the rate-limiting reaction. The fact that the metabolic flux through the subpopulation of normal or genetically reconstituted cells cocultivated with *pccA* cells is greater than normal suggests that metabolic cooperation can provide the limiting substrate for reactions in metabolically competent cells.

These data illustrate how the potential for somatic gene therapy raises novel questions concerning holoenzyme assembly, the kinetics of metabolic flux, and the metabolic interactions among cells. The challenge of understanding the consequences of genetic reconstitution may prove to be much like those facing metabolic engineering in simple organisms, where the presence of accessory pathways and metabolic rigidity often confound efforts to attain predictable ends (Bailey 1991; Stephanopoulos and Vallino 1991).

Acknowledgments

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Propionate Metabolism in Cultured Human Cells after Overexpression of Recombinant Methylmalonyl CoA Mutase: Implications for Somatic Gene Therapy

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Abstract—*Strategies for somatic gene therapy must consider the metabolic consequences of expressing the recombinant gene product in addition to methods for gene transfer and expression. We describe studies of propionate metabolism in cultured cells transfected with methylmalonyl CoA mutase (MCM), the enzyme deficient in* mut *methylmalonic acidemia. Transfection of MCM into* mut *fibroblasts restores propionate metabolism to normal levels in a dose-dependent manner. Overexpression of MCM, or the addition of excess propionate, carnitine, or cobalamin, does not increase propionate metabolism in normal human fibroblasts, lymphoblasts, or hepatoma cells, although hepatic cells exhibit >10-fold higher levels of propionate metabolism. Significantly, the restoration of propionate metabolism in* mut *fibroblasts is disproportionately greater than the efficiency of transfection, suggesting the presence of a cooperative phenomenon between cells. Intercellular participation in propionate metabolism is evident in cocultures of MCM-deficient and propionyl CoA carboxylase-deficient cells. We conclude that the liver is the preferred target for gene therapy of MCM deficiency because of its greater capacity for propionate metabolism and that cooperation between cells could enhance the biological effect of a subpopulation of cells transformed with recombinant MCM.*

INTRODUCTION

Metabolism of propionate absorbed from the gastrointestinal tract, or propionyl CoA generated from intermediary metabolism, involves a series of mitochondrial matrix enzymes, including methylmalonyl CoA mutase (MCM, EC 5.4.99.2) and propionyl CoA carboxylase (PCC, EC 6.4.1.3) (1–3). Inherited deficiencies of these enzymes, including methylmalonic acidemia (MMA, MCM deficiency, McKusick # 251000) or propionic acidemia (PCCA McKusick # 23200; PCCB deficiency, McKusick # 23205) are associated with severe disruptions in organic acid, amino acid, carbohydrate, and ammonia metabolism. These disorders continue to be associated with profound morbidity and significant mortality despite dietary, vitamin, and carnitine therapy (2).

The development of methods for somatic gene therapy (4–6), and the commencement of clinical trials involving gene transfer into human subjects (7), has introduced the possibility of considering somatic gene therapy for many inherited metabolic diseases, such as MMA (8). We have reported cloning cDNAs for human and murine MCM (9–11)

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and the restoration of normal propionate metabolism in MCM deficient (*mut*) fibroblasts by DNA-mediated gene transfer (11, 12). Recently, we have developed retroviral vectors that are capable of correcting the defect in primary MCM-deficient fibroblasts by viral-mediated gene transfer (13). These vectors can also be used to efficiently introduce MCM into primary human hepatocytes and direct expression of the recombinant gene. We have also demonstrated that recombinant MCM can be introduced into the livers of experimental animals by DNA-mediated gene transfer, using asialoglycoprotein-coupled DNA (Stankovics, Wu, and Ledley, unpublished data). These results, together with the initiation of clinical trials to test methods for gene delivery to the liver (14), suggest it will be technically possible to introduce normal MCM into a population of cells in human subjects in order to treat *mut* MMA.

In developing rational schemes for somatic gene therapy, it is necessary to consider not only how to attain adequate expression of a functional apoenzyme, but also the adequacy of the attendant steps required for metabolism of the substrate to suitable metabolic products (8). Thus, it is necessary to consider whether genetically reconstituted cells will provide the substrate for the recombinant enzyme and eliminate its product, whether the level of recombinant enzyme activity will be rate limiting, and whether metabolism in cells that are not genetically reconstituted will be affected by a subpopulation of metabolically competent cells. These considerations will determine which somatic cell type is the appropriate target for somatic gene therapy, what levels of recombinant gene expression are required to optimally restore metabolism, and how many cells need to be transduced in order to restore metabolic homeostasis.

These questions are difficult to answer because they involve novel, nonphysiological situations, which are not necessarily predictable from conventional understandings of homogeneous in vivo systems where every cell expresses the same genotype. Ideally these questions could be answered by experimental gene therapy in homologous animal models for inherited disease. There is, however, no genetic animal model of isolated MCM deficiency, and animal models of cobalamin deficiency are complicated by concomitant defects in methionine synthase activity (15, 16).

In the absence of an adequate animal model for MMA, cultured cells represent the best available system for studying the response of cells to overexpression of recombinant MCM. This report describes studies in several cell types that are commonly considered potential targets for somatic gene therapy (6).

MATERIALS AND METHODS

Cell Lines. Primary MCM-deficient fibroblasts (*mut*) GM50 and GM1673 were obtained from the NIGMS mutant cell repository and have been characterized previously (17). Primary PCC deficient fibroblasts PCC756 were kindly provided by Dr. Wayne Fenton (Yale University). Fibroblasts were grown in MEM with 20% fetal bovine serum (JRH Bioscience, Lenexa, Kansas) and supplemented with essential amino acids and vitamin solutions (Gibco-BRL, Gaithersburg, Maryland). The human hepatoma cell lines PLC/PRF and hep3b2 (18) were grown in Waymouth's/MEM 3:1 mixture with 10% fetal bovine serum. Lymphoblasts (Bab-4) were kindly provided by Dr. Pragna Patel (Baylor College of Medicine) and grown in F12 media with 10% fetal bovine serum. Primary human hepatocytes were harvested and cultivated as described previously (13, 14).

Expression Vectors and Electroporation. Expression vectors used in this study have been described previously: the *E. coli* βgal gene (PCMV-βgal) (19) or the murine MCM

cDNA (pCMV-mMCM) expression vector (11, 12). Electroporation was performed using a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Richmond, California) (20), as described previously (11) using 0.3 ml of cells at a density of 3×10^6–10^7 cells/ml, 0.1–70 μg of DNA, and voltage pulses of 220–280 V at a capacitance of 500 μF or 960 μF. MCM activity was assayed two days after electroporation during the transient phase of gene expression.

Measures of Methylmalonyl-CoA Mutase Enzyme Activity and Propionate Metabolism. Three assays of MCM activity or propionate metabolism were used in this study: *(1)* An in vitro assay for MCM was used to assess the level of expression of this enzyme in transfected cells and controls. *(2)* An assay of [^{14}C] propionate incorporation into trichloroacetic acid (TCA)-precipitable material is commonly used as a measure of propionate metabolism via PCC and MCM to amino acids. Acid soluble material from cultured cells includes free fatty acids and acyl-carnitine derivatives (16, 21). *(3)* An assay of propionate concentration in fresh and conditioned media by HPLC (22) provides a measure of the total flux of propionate to metabolic products, including oxidation to CO_2. Similar data are obtained by assaying the release (loss) of ^{14}C counts from cultures containing [^{14}C]propionate.

MCM activity is measured in vitro by the conversion of [^{14}C]methylmalonyl-CoA to [^{14}C]succinyl-CoA using the potassium permanganate–perchloric acid method described previously (17, 23). Assays were performed with [methylmalonyl CoA] = 0.5 mM. All experiments were performed in triplicate and data are expressed as the mean with standard deviations commonly <3% of the mean.

Incorporation of [^{14}C]propionate into TCA-precipitable material was determined as described previously (17, 24, 25). Confluent cells in 12-well plates were incubated for 18 h in 0.5 ml Puck's-G saline with 6×10^4 dpm of [^{3}H]leucine/ml (130 Ci/mmol; Amersham, Arlington Heights, Illinois) ([leucine] = 37 μM), 2×10^6 dpm of [1-^{14}C]propionate/ml (57 mCi/mmol; New England Nuclear, Boston, Massachusetts), 100 μM propionate, 15% (v/v) fetal bovine serum, 1.2 mg of $NaHCO_3$/ml, 8.8 mg of glucose/ml, and 1.0 μg of hydroxocobalamin/ml. The incorporation of ^{14}C and ^{3}H into the TCA-precipitable fraction was determined by washing the cells three times in 0.13 M NaCl–18 mM sodium phosphate, pH 8.0, (PBS) followed by three times for 15 min with 5% (w/v) TCA at 4°C and resuspending precipitated material in 1 ml 0.2 M NaOH for counting in Aquasol (New England Nuclear, Boston, Massachusetts) with correction for dual ^{14}C and ^{3}H standards. In this experiment ^{3}H equilibrated in the protein pool is used as a denominator for cell number and active protein synthesis. The first TCA wash was recovered and counted as a measure of metabolites in the acid-soluble fraction. All experiments were performed in triplicate, and data are expressed as the mean. Standard deviations were commonly below 5% of the mean, unless shown differently. Protein determination was performed by the Bradford procedure (Bio-Rad Laboratories, Richmond, California).

The total flux of [^{14}C]propionate was assessed by measuring the concentration of propionate in fresh media, conditioned media, and control media incubated without cells using an HPLC assay for organic acids (22). This assay uses an isocratic HPLC (Waters Associates, Milford, Massachusetts), an ORH-801 organic acid chromatographic column, 30 × 0.38 cm (ID) (Interaction Chemicals, Mountain View, California), and a model 431 conductivity detector connected to an on-line radioactivity detector (Flo-One Beta, Radiomatic, Packard Instruments Co., Downers Grove, Illinois). The mobile phase, a 0.4 mN solution of "ultrapure" sulfuric acid in Milli-Q-purified

water (background conductance <100 μS), was filtered through a 0.2-μm (pore size) membrane and degassed before use. Separation of volatile fatty acids (C_2–C_5) was best with a flow rate of 0.5 ml/min and a column temperature of 37°C. A standard calibration curve was determined using formate, acetate, propionate, butyrate, isovalerate, and valerate (Alltech, Deerfield, Illinois). Media with an internal standard (butyrate) was filtered and injected into the HPLC. Data shown are the average of duplicate HPLC determinations and are expressed as the difference between the concentration of propionate in control media incubated 18 h at 37°C in the absence of cells and the total concentration of propionate remaining in conditioned media (expressed as nanomoles propionate per milligram of cellular protein). Standard deviations were typically around 5–10% of the mean. Alternatively, the loss of ^{14}C from the media during the period of cultivation was determined by counting ^{14}C in fresh and conditioned media. Aliquots of media from each well of a 12-well plate were counted using Aquasol. Loss of ^{14}C was determined by subtracting the total remaining counts in the conditioned media from the total counts in media incubated in wells without cells. This value correlated well with determinations of propionate concentration.

Cocultivation Methods. For cocultivation, cells were plated on 12-well plates at a density of 150,000 cells/well. For some experiments cells were pretreated with mitomycin-C (Sigma Chemical Company, St. Louis, Missouri) at a concentration of 5 μg/10^6 cells. For membrane experiments 10^5 cells were plated on Falcon Cell Culture Inserts (Becton Dickinson Labwares, Lincoln Park, New Jersey) and suspended over 1.5×10^5 cells plated on the plastic surface.

RESULTS

Overexpression of Recombinant MCM in Primary Fibroblasts and Hepatoma Cells. Electroporation was used to introduce recombinant MCM cDNA under the transcriptional control of the cytomegalovirus (CMV) immediate early promoter/enhancer into fibroblasts, lymphoblasts, and hepatoma cells. Protein extracts were prepared 48 h after electroporation during the transient phase of gene expression and assayed for MCM enzyme activity (Table 1). Electroporation of normal fibroblasts resulted in a fivefold increase in assayable enzyme activity. Electroporation of PLC/PRF human hepatoma cells led to a twofold increase in enzyme activity. Electroporation of MCM deficient *mut* fibroblasts resulted in constitution of MCM enzyme activity at levels significantly higher than normal fibroblasts. These experiments demonstrate overexpression of functional MCM in transfected cells.

Effect of MCM Overexpression on Propionate Metabolism. The incorporation of ^{14}C from propionate to TCA-precipitable material is commonly used as a measure of propionate metabolism by primary fibroblasts. This incorporation is dependent upon MCM and PCC activities as evidenced by the absence of significant incorporation of [^{14}C]propionate into TCA-precipitable material in MCM- or PCC-deficient cells (Fig. 1A). We used two additional measures of propionate metabolism to account for the flux of propionate to products that were not TCA-precipitable, the major concern being

Table 1. Overexpression of MCM in Fibroblasts and Hepatoma Cells after Electroporation with Recombinant MCM

	Enzyme activity[a]	
	Basal	Transfected[b]
Normal fibroblasts	40[c]	191[c]
mut fibroblasts	0 (10)	912 (44)
PLC/PRF hepatoma cells	495 (53)	990 (42)

[a]Succinate formed (nmol/mg protein/h); data are the mean and standard deviation of triplicate samples.
[b]Cells were transfected by electroporation using 11×10^6 cells, 20 μg of the mMCM expression vector, pCMV-mMCM$_5$, 260 V at a capacitance of 960 μF.
[c]Data from Wilkemeyer et al. (12), assay with [methylmalonyl-CoA] = 0.3 mM. Standard deviation was less than 5% of the reported value.

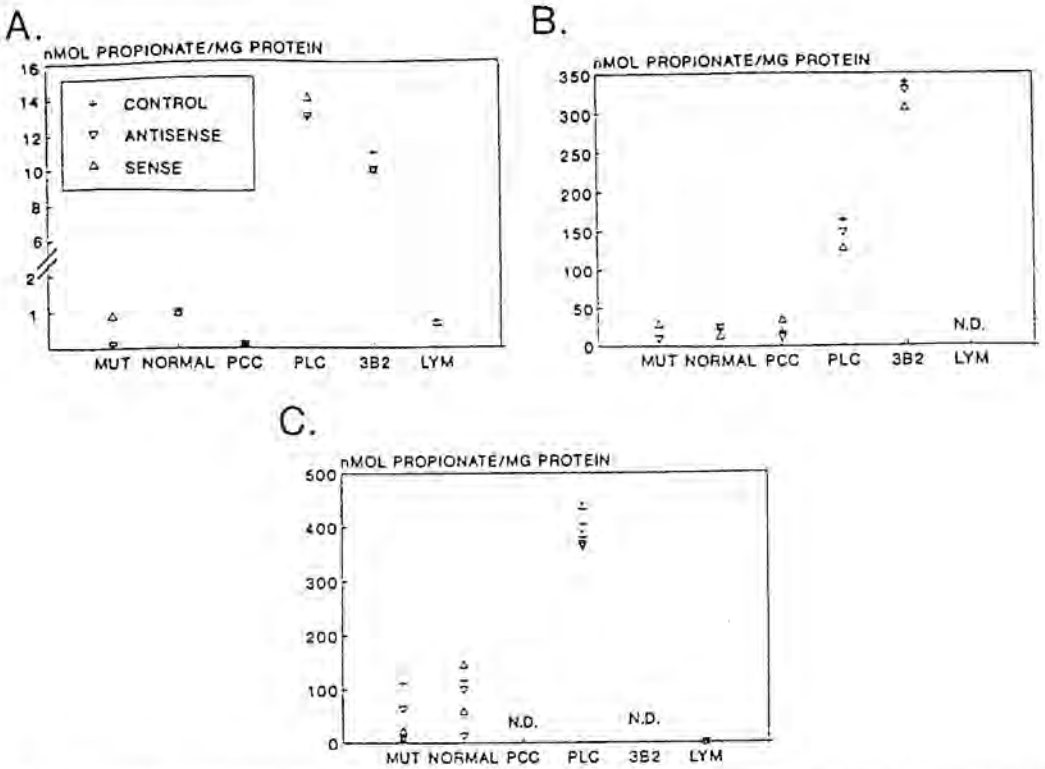

Fig. 1. Propionate metabolism in cultured human cells before and after overexpression of recombinant MCM. (A) Measurement of [^{14}C]propionate incorporation into TCA-precipitable material. This measures metabolism of propionate via MCM to amino acids. (B) Release of ^{14}C from conditioned media. This measures metabolism of propionate to more volatile products, specifically CO_2. (C) Total catabolism of propionate determined as the difference in the concentration of propionate in fresh media and media conditioned on cultured cells (expressed as nmol propionate/mg cellular protein). Propionate concentrations were determined by HPLC. For these experiments electroporations were carried out with 0.15 ml of cells (density, 3×10^6/ml), using 10 μg DNA with a voltage of 260 V at a capacitance of 960 μF. Data in A and B are expressed as the mean of triplicate samples. Individual data points of duplicate experiments are shown in C. Legend: (+) transfected with control vector (pCMV) consisting of the empty expression vector; (△) transfected with expression vector pCMV-mMCM, containing the mouse MCM cDNA in the sense orientation; (▽) transfected with expression vector pCMV-mMCM$_{AS}$ containing the mouse MCM cDNA in anitsense orientation. N.D., not determined; MUT, *mut*^{0} fibroblasts; NORMAL, normal fibroblasts; PCC, *pcc* fibroblasts; LYM, normal human lymphoblasts; PLC, PLC/PRF hepatomas; and 3B2, Hep3B2 hepatomas.

oxidation of propionate to CO_2, which is the major end product of hepatic metabolism. The catabolism of propionate was measured by the loss of ^{14}C counts from media after incubation with cultured cells (Fig. 1B) and by HPLC determination of propionate concentrations in fresh and conditioned media (Fig. 1C). Sample variability was significantly higher with the HPLC determination. Incorporation of ^{14}C into TCA-precipitable material was 10-fold higher in hepatoma cells than in primary fibroblasts. The TCA-precipitable fraction in hepatoma cells accounted for only 3–10% of the total propionate catabolism evident by HPLC determination of propionate remaining in conditioned media or ^{14}C loss from the media. Measures of propionate metabolism in primary human hepatocytes were very similar to observations in hepatoma cells (data not shown).

We have previously observed that transfer of recombinant MCM into primary MCM-deficient fibroblasts restores propionate incorporation to normal levels (11, 12). The data in Fig. 1A confirm this observation. These data also demonstrate that overexpres-

sion of normal MCM in normal fibroblasts, normal lymphoblasts, or PLC/PRF hepatoma cells does not significantly increase either the fraction of [^{14}C]propionate metabolized to TCA-precipitable material (Fig. 1A) or the total catabolism of propionate (Fig. 1B and C). The fact that overexpression of MCM does not affect propionate flux in normal fibroblasts, lymphoblasts, or hepatoma cells suggests that propionate metabolism is not limited by the level of the MCM enzyme in these cells.

Propionate Metabolism as a Function of Electroporation Efficiency. To determine the relationship between the level of MCM expression and reconstitution of propionate metabolism in *mut* fibroblasts, we assayed propionate incorporation after electroporation with different concentrations of the MCM expression vector (Fig. 2) and using different conditions for electroporation (Fig. 3). The number of copies of the expression vector introduced into transfected cells can be varied by using constant electroporation conditions and diluting the expression vector with an identical construct lacking the MCM gene. The fraction of cells taking up DNA is a function of the conditions used for electroporation (20) and can be varied by changing the voltage.

Propionate incorporation increased as a function of the amount of expression vector used in the electroporation when the total amount of DNA was maintained at 20 μg by addition of the empty CMV vector or when the expression vector was electroporated alone at different concentrations (Fig. 2A). Electroporation of normal fibroblasts with the expression vector and electroporation of MCM-deficient cells with the antisense vector had no effect on propionate incorporation. To demonstrate the relationship be-

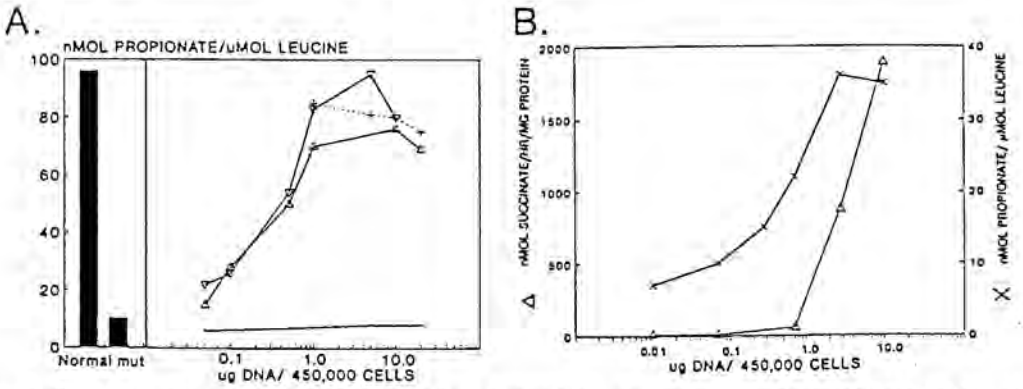

Fig. 2. Propionate incorporation in *mut* fibroblasts induced by electroporation of recombinant MCM. (A) Propionate incorporation in *mut* fibroblasts in response to electroporation with different concentrations of the MCM expression vector. Electroporations were performed by either diluting different concentrations of the pCMV-mMCM$_S$ (MCM cDNA in the sense orientation) plasmid with empty expression vector for a constant DNA concentration of 20 μg (▽) or with different concentrations of pCMV-mMCM$_S$ plasmid alone (△). Identical electroporations in *mut* cells were done with different concentrations of an antisense clone, pCMV-mMCM$_{AS}$ (■), or in normal fibroblasts with different concentrations of the pCMV-mMCM$_S$ plasmid (+). Values for nonelectroporated normal and *mut* cells are shown by bars on the left. [^{14}C]Propionate incorporation into TCA-precipitated counts was measured for each condition and is expressed as the mean of triplicate samples. Standard deviation was less than 5% of the mean. Electroporations were carried out with 0.15 ml of cells at 3 × 10^6/ml, using 0.05–20 μg DNA with voltage pulses of 260 V at capacitance of 960 μF. (B) Correlation of MCM enzyme activity (△, left axis) and [^{14}C]propionate incorporation (X, right axis) after transfection with different concentrations of the MCM expression vector. [^{14}C]Propionate incorporation into TCA-precipitated counts was measured for each condition and is expressed as the mean of triplicate samples. MCM enzyme activity is expressed as the average of two samples determined on different days; standard deviation was less than 10% of the mean. Electroporations were carried out with 0.3 ml of cells at 11 × 10^6/ml, using 0.07–70 μg DNA with voltage pulses of 260 V at capacitance of 960 μF.

tween the DNA concentration used in electroporations, the amount of functional recombinant enzyme expressed, and reconstitution of propionate incorporation, this experiment was repeated using scaled-up electroporation conditions (Fig. 2B). Results are normalized to the amount of DNA per cell in the electroporation enabling a comparison with data in Fig. 2A. Enzyme activity increased as a function of the amount of DNA in the electroporation. Significant propionate incorporation was evident after electroporation with as little as 0.6 μg of DNA/450,000 cells. At these conditions, the enzyme is barely detectable with the in vitro assay (Fig. 2B).

To evaluate the effect of electroporation efficiency (fraction of cells transfected), experiments were performed with voltages ranging from 220 to 280 V at a constant capacitance of 960 μF (Fig. 3). Control electroporations were performed using a vector containing the βgal gene, and the fraction of cells transfected with this plasmid was scored by staining with X-gal. The fraction of X-gal-positive cells varied with voltage and ranged from 10% at 220 V to 55% at 280 V (Fig. 3). In experiments performed under identical conditions using the MCM expression vector, there was only a fractional increase in the level of propionate incorporation over this same range. There was no change in the TCA-precipitable fraction from normal cells electroporated with the expression vector or *mut* cells electroporated with an antisense construct as controls. These data demonstrate that the restoration of propionate metabolism was disproportionately greater than the fraction of cells transfected.

Cocultivation of Normal and Mutant Cells. We considered the possibility that this apparent disproportionality between the reconstitution of MCM enzyme activity and rate of propionate metabolism was an artifact related to the transient, electroporation system. Several potential problems were

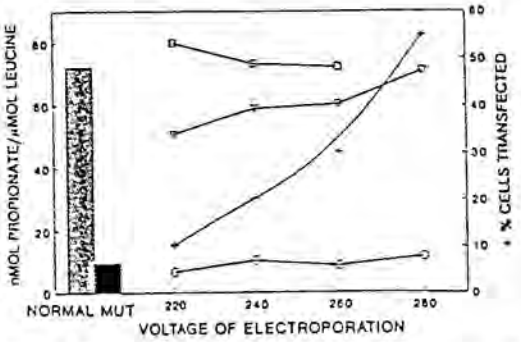

Fig. 3. Propionate incorporation as a function of transfection efficiency (voltage of electroporation). We compared the effect of electroporation efficiency, measured by transfection with a pCMV-βgal marker gene, with the level of [^{14}C]propionate incorporation restored by transfection with pCMV-mMCM$_S$. The fraction of cells expressing βgal was scored by staining with X-gal and cell counting (+, right axis). Electroporations were performed using identical conditions with the pCMV-mMCM$_S$ clone into normal cells (□, left axis) or *mut* cells (▽, left axis) and with the pCMV-mMCM$_{AS}$ clone in *mut* cells (○, left axis). Values for nonelectroporated normal and *mut* cells are shown by bars at left. [^{14}C]Propionate incorporation into TCA-precipitated counts was measured for each condition and is expressed as the mean of triplicate samples. The standard deviation was typically less than 5% of the mean. Electroporations were carried out with 0.15 ml of cells (density, 3×10^6/ml), using 10 μg DNA with voltage pulses from 220 to 260 V at a capacitance of 960 μF.

recognized: *(1)* underestimation of the true frequency of transfection with the X-gal histochemical stain, *(2)* overexpression of MCM from the CMV promoter in transfected cells, *(3)* disruption of normal cell structure by electroporation, or *(4)* unequal proliferation of transfected and nontransfected cells. To control for these variables, we performed identical experiments in which normal fibroblasts were cocultivated with *mut* fibroblasts in different ratios. We assayed propionate incorporation in these cocultures as well as in cocultures of cells pretreated with mitomycin C control for possible differential growth rates between the cell types.

Table 2 shows the level of propionate incorporation as a fraction of maximal activity versus the fraction of normal cells

Table 2. Disproportional Propionate Incorporation in Mixtures of Normal and *mut* Fibroblasts.

	Percentage of normal cells in culture[a]	
	10%	30%
Percent of maximal activity		
(+) Mitomycin C	30	70
(−) Mitomycin C	40	62

[a] If there were no cooperation between cells, the percent maximal activity would equal the percentage of normal cells in culture. Measurement of [^{14}C] propionate incorporation into the TCA-precipitable fraction was determined for each condition.

included in the cocultivation. These data show that addition of 10–30% normal cells to the mitomycin C cocultures results in a disproportionately greater reconstitution of propionate incorporation, similar to that observed in transfection experiments. The same overall trend was seen when cells were not pretreated with mitomycin C. Significantly, the total propionate flux exhibited by mixed cultures is never observed to exceed the flux of control plates containing only normal fibroblasts.

Propionate Flux in Cocultures of mut *and* pcc *Cells.* We postulated that this disproportionality could result from a cooperative phenomenon between cells in which metabolites accumulating in MCM-deficient cells moved into MCM-competent cells and metabolized via MCM. This could increase the amount of substrate available for MCM in these cells. To demonstrate that such cooperation could occur, we investigated propionate metabolism in cocultures of *mut* and *pcc* fibroblasts in the absence of cell fusion (Fig. 4A). While neither *mut* nor *pcc* fibroblasts are capable of significant propionate incorporation, we predicted that if methylmalonyl-CoA or its derivatives produced by carboxylation in *mut* cells were capable of moving into *pcc* cells, then cocultivation would result in detectable propionate incorporation. Cocultivation, in fact, resulted in levels of

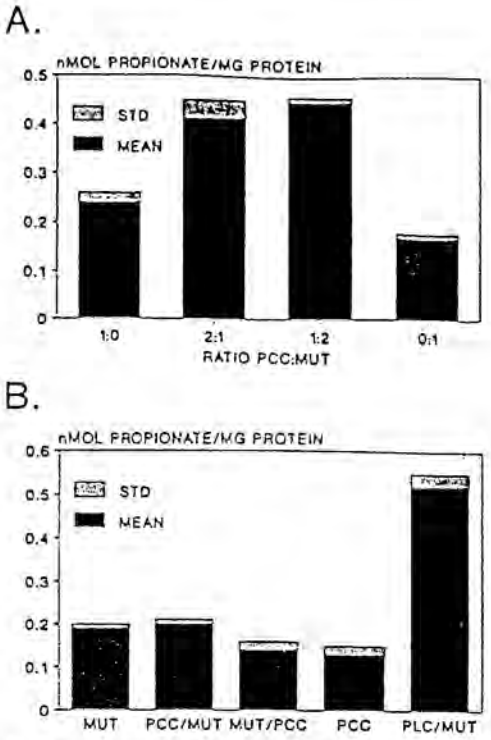

Fig. 4. Propionate metabolite transfer between *mut* and *pcc* cells. (A) Propionate incorporation in cocultivations of *mut* and *pcc* cells. This combination of mutant cells was chosen because neither cell alone is capable of any substantial propionate incorporation. Different proportions of *mut* and *pcc* cells or parental cells alone were cocultivated and [^{14}C]propionate incorporation into the TCA-precipitable fraction was measured (see Materials and Methods for details). The mean and standard deviation (STD) of triplicate samples are indicated. MUT, *mut* fibroblasts; PCC, *pcc* fibroblasts; and PLC, PLC/PRF hepatomas. (B) Propionate incorporation in cocultivations of cells separated by a membrane. Cells were grown on tissue culture plates or on Falcon Cell Culture Inserts suspended over the cells on the plate (see Materials and Methods for details). [^{14}C]Propionate incorporation into the TCA-precipitable fraction was only measured for cells on the plate either alone (MUT, PCC) or after cocultivation with cells on the culture inserts, i.e., *pcc* cells on the insert and *mut* cells on the plate, (PCC/MUT). The mean and standard deviation (STD) of triplicate samples are indicated. MUT, *mut* fibroblasts; PCC, *pcc* fibroblasts; and PLC, PLC/PRF hepatomas.

propionate incorporation two to threefold higher than either parental cell line (Fig. 4A), demonstrating that metabolites are capable of moving between cells. Similar

cocultivation experiments (without cell fusion) between the different genotypic classes of *cbl* mutations also demonstrated up to a twofold increase over expected values for propionate incorporation (26).

We considered whether this cooperative phenomenon could occur across a fluid phase, which would allow us to identify the metabolites participating in this process. For these experiments, one fibroblast line was grown on cell culture-treated membranes over a tissue culture surface containing the other fibroblast line, and propionate incorporation was measured in the cells grown on the plates using standard methods (Fig. 4B). No change in propionate incorporation was observed when *mut* fibroblasts were grown over *pcc* fibroblasts or *pcc* fibroblasts were grown over *mut* fibroblasts. In contrast, when hepatoma cells were grown on the membrane over *mut* cells there was a two- to threefold increase in ^{14}C incorporation in the mutant fibroblasts (Fig. 4B). However, there is no evidence that this increase involves intermediates of propionate metabolism since hepatomas are capable of synthesis of a wide variety of end products, including glucose and volatile fatty acids, which could be taken up by fibroblasts.

To determine whether the effect of

Table 3. Propionate Metabolism in Cultured Cells with or without Addition of Carnitine at Two Concentrations of Propionate[a]

	0.1 mM propionate				2 mM propionate			
	TCA precip		TCA soluble		TCA precip		TCA soluble	
	Mean	(SD)	Mean	(SD)	Mean	(SD)	Mean	(SD)
Normal–primary fibroblasts								
	1.0	(0.03)	0.3	(0.04)	4.3	(0.18)	3.8	(0.21)
	1.1	*(0.06)*	*1.1*	*(0.09)*	*4.8*	*(0.48)*	*6.6*	*(0.41)*
mut–primary fibroblasts								
	0.2	(0.03)	0.4	(0.03)	0.6	(0.04)	1.3	(0.16)
	0.2	*(0.01)*	*3.4*	*(0.10)*	*0.7*	*(0.02)*	*7.8*	*(0.12)*
pcc–primary fibroblasts								
	0.2	(0.01)	0.5	(0.02)	0.6	(0.14)	3.5	(0.59)
	0.1	*(0.01)*	*2.6*	*(0.17)*	*0.6*	*(0.05)*	*10.8*	*(0.97)*
PLC–human hepatoma								
	21.5	(0.18)	2.2	(0.12)	71.4	(0.15)	18.3	(1.40)
	19.6	*(0.82)*	*2.0*	*(0.02)*	*80.7*	*(1.20)*	*31.6*	*(0.16)*
Normal + *mut*								
	1.9	(0.06)	1.0	(0.08)	5.1	(0.13)	3.4	(0.37)
	1.8	*(0.09)*	*3.8*	*(0.11)*	*5.6*	*(0.21)*	*13.2*	*(0.14)*
pcc + *mut*								
	0.5	(0.05)	0.5	(0.03)	1.6	(0.11)	1.8	(0.30)
	0.4	*(0.04)*	*4.2*	*(0.41)*	*1.9*	*(0.15)*	*12.8*	*(0.66)*
pcc/*mut*								
	0.2	(0.01)	0.4	(0.03)	0.6	(0.06)	1.2	(0.03)
	0.2	*(0.01)*	*3.1*	*(0.17)*	*0.9*	*(0.03)*	*7.0*	*(0.38)*
mut/*pcc*								
	0.2	(0.01)	0.5	(0.03)	0.6	(0.02)	4.0	(0.10)
	0.2	*(0.01)*	*2.1*	*(0.25)*	*0.8*	*(0.20)*	*8.8*	*(1.20)*
PLC/*mut*								
	0.5	(0.03)	0.6	(0.01)	2.4	(0.45)	2.5	(0.23)
	0.6	*(0.03)*	*4.0*	*(0.07)*	*2.8*	*(0.59)*	*10.2*	*(0.26)*

[a]The distribution of ^{14}C-labeled products in the TCA-precipitatable (TCA precip) and -soluble (TCA Soluble) fractions was measured and calculated as nanomoles propionate per milligram (cellular) protein. The second row of numbers (*italics*) for each column are the data points for addition of carnitine; 5 mM. Data are expressed as the mean of triplicate samples with standard deviation in parenthesis.

cocultivating *mut* and *pcc* cells would be enhanced by high concentrations of propionate such as those observed in MMA or PA, propionate incorporation was assayed with concentrations of propionate up to 2 mM (Table 3). High concentrations of propionate increased incorporation into TCA-precipitable material in both *mut* and *pcc* cell lines, reflecting nonspecific background or utilization of alternative pathways for propionate metabolism (27). Propionate incorporation of cocultivated *mut* and *pcc* increased proportionately with their basal activity. There was no evidence of increase propionate incorporation when the *mut* and *pcc* cells were separated by a fluid phase on membranes. We also considered whether carnitine would stimulate propionate flux or the movement of metabolites between cells. Treatment with L-carnitine has been shown to enhance the formation and excretion of short-chain acylcarnitines in patients with PA and MMA (28). Carnitine was added to cultures at concentrations up to 5 mM (Table 3, italics) but had no effect on propionate incorporation. This is consistent with previous observations that addition of carnitine to cultured rat hepatocytes increased the formation of propionylcarnitine but not the level of CO_2 or glucose produced from labeled propionate (15, 16, 29).

DISCUSSION

In developing sound strategies for somatic gene therapy of metabolic diseases, it is necessary to consider not only efficient methods for transduction and transcription of the recombinant gene, but also the metabolic consequences of recombinant gene expression. Recombinant enzymes will only have biological effects if cells are able to provide substrate and cofactors, eliminate metabolic products, and provide heterologous or regulatory subunits without interfering with other essential cellular functions (8). These issues are analogous to those facing metabolic engineering in microorganisms, where shortcomings in accessory pathways and inherent metabolic rigidity have commonly interfered with efforts to enhance production of normal metabolites or engineer production of novel compounds (30, 31). This experience in microorganisms is relevant to gene therapy because it illustrates the limitations of a priori reasoning in predicting the biological consequences of recombinant engineering.

The present work describes studies in cultured cells aimed at developing a rational approach to somatic gene therapy of MMA. Our goal was to evaluate which somatic targets were appropriate for gene therapy, to consider what levels of recombinant gene expression would be required to constitute optimal metabolic activity, and to investigate whether there was any evidence that a subpopulation of metabolically corrected cells would affect propionate metabolism in surrounding cells that would remain genetically defective. This work made use of transient expression systems in which the normal MCM gene was electroporated into cells and expressed for several days before the cells were harvested for analysis. While transient expression is commonly used as a model for assessing transcriptional processes, there is little experience with the effects of transient gene expression on the metabolic function of cultured cells. We chose to use transient expression because this method offers considerable versatility in the ability to alter the fraction of cells transformed with the recombinant gene and the level of gene expression. Moreover, the level of gene expression during transient expression is highly reproducible between experiments, since it is not dependent upon position effects related to the site of integration.

There are presently no genetic models for *mut* MMA. In vivo models of MCM deficiency induced by B_{12} deprivation or B_{12} inhibitors are complicated by the concomi-

tant deficiency of methionine synthase (16) and recombinant MCM would not be active in these cells in the absence of cofactor. While we are investigating the possibility of generating a MCM-deficient mouse model by homologous recombination (32), the present work has necessarily focused on primary human fibroblasts that can be easily obtained from patients. Studies in primary human fibroblasts are seriously limited by the low efficiency of stable transformation after DNA-mediated gene transfer and the inability to adequately expand rare clones of cells to high passage numbers.

Our data suggest that the liver will be the preferred target for somatic gene therapy. Human hepatoma cells and primary hepatocytes exhibit levels of propionate flux > 10-fold higher than either normal primary fibroblasts or lymphoblasts, which are commonly considered targets for gene therapy. Moreover, overexpression of recombinant MCM, increasing the concentration of propionate to the levels seen in pathological conditions (up to 2 mM), and administration of pharmacological doses of hydroxycobalamin (data not shown) or pharmacological levels of carnitine did not increase the metabolic capacity of nonhepatic cells to the level exhibited by hepatoma cells or primary hepatocytes.

The fact that propionate metabolism of normal cells is not increased by these manipulations illustrates that propionate flux is not normally limited by the level of MCM enzyme. It should be noted that there is no evidence that the rate limiting step has to be the same in different cell types. The greater capacity for propionate flux in hepatic cells indicates only that the rate-limiting activity is constituently higher in hepatic cells than fibroblasts or lymphoblasts. We have not addressed the nature of the rate-limiting reaction component in this study.

The critical point with regard to somatic gene therapy is that without being able to affect the rate-limiting component of this pathway, it is unlikely that reconstitution of MCM in nonhepatic cells would constitute the same metabolic capacity as transduction of hepatocytes. Thus, we conclude that the metabolic effect of reconstituting MCM activity in the liver would be greater than reconstituting this activity in fibroblasts of lymphocytes. Hepatic reconstitution of MCM would also most closely restore physiological metabolism of propionate, since the liver is the principle site of catabolism of propionyl-CoA derived from intermediary metabolism or propionate absorbed from the gut (3).

The observation that overexpression of recombinant MCM does not induce an increase in propionate flux through normal cells also suggests that somatic gene therapy may not require a particularly high level expression of MCM and would not be enhanced by overexpression of the recombinant gene. In fibroblasts, maximal propionate metabolism requires a relatively low level expression of MCM. Moreover, studies with mutant MCM genes demonstrate that propionate incorporation can be detected after gene transfer with mutants that produce essentially undetectable levels of the MCM enzyme (33, 34). This is consistent with the previous observation that propionate flux can be restored in *cbl* mutant cells by restoring the level of MCM holoenzyme to approximately 10% of normal (35).

The most intriguing observation in the present data is that propionate metabolism by cocultures of mutant and metabolically competent fibroblasts is consistently greater than the sum of activity expected from the subpopulation of competent cells. This phenomenon has also been observed in primary fibroblasts stably transformed by retroviral transduction with a vector expressing human MCM (13). This is paradoxical in light of the inability to increase propionate metabolism by the measures described above. This observation is, however, consistent with a hypothesis that the rate-limiting reaction in fibroblasts involves steps in derivation of

methylmalonyl-CoA from propionate and that products of this rate-limiting reaction are able to move between mutant and metabolically competent cells. In this model, the rate of propionate catabolism through MCM in the individual metabolically competent cells would be greater than normal, since more substrate for MCM would be available from metabolism occurring in adjacent cells. The metabolic capacity of the entire culture, however, would remain limited by the capacity of the rate-limiting reaction and would be no greater than that of normal cultures. While the present experiments do not explicitly demonstrate this model, we show that movement of intermediates can occur by documenting limited reconstitution of propionate incorporation in cocultivated *mut* and *pcc* cells. In this experiment, propionate incorporation requires the movement of methylmalonyl-CoA (or a derivative) from MCM-deficient cells into PCC-deficient cells where it could be metabolized by MCM. This observation, suggesting a cooperative phenomenon between adjacent cells, may enable catabolism of metabolic products accumulating in deficient cells. We have not been able to test the hypothesis that this phenomenon represents metabolic cooperation in its strictest sense involving transport of metabolites through gap junctions between cells (36).

The phenomenon we observed in cocultivated *mut* and *pcc* cells is analogous, but distinct, from that observed in cocultivation of certain cells lines with mutations in cobalamin metabolism (26). Cocultivation of fibroblasts from *cblC* and *cblF* genotypes resulted in a twofold increase in [^{14}C]propionate incorporation, presumably involving the exchange of cobalamin derivatives, while other combinations of the seven *cbl* cell lines (*cblA*, *cblB*, *cblC*, *cblD*, *cblE*, *cblF*, and *cblG*) showed little or no effect.

Several laboratories have described strategies for somatic gene therapy of the liver that involve partial hepatectomy, cultivation of primary hepatocytes in vitro, transduction with recombinant retroviral vectors, and autologous transplantation of these genetically engineered cells into the liver via the portal circulation or spleen (14, 37–42). This approach to somatic gene therapy would result in genetic reconstitution of only a fraction of hepatic cells. Studies in animal models suggest that with existing gene transfer and transplantation technologies, this fraction will be $<2\%$ of hepatocytes (14).

The present data do not allow us to conclude whether this would be quantitatively sufficient to alter the pathological phenotype of the disease. These data do suggest, however, that subpopulations of genetically reconstituted cells in vitro could have a disproportional corrective effect on propionate metabolism. It remains to be demonstrated that a similar cooperative phenomenon will be operative between stably transformed hepatic cells in vivo. If so, then it is reasonable to expect that somatic gene therapy of a fraction of hepatic cells may have a significant phenotypic and clinical impact.

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III.számú közlemény

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Overexpression of Human Methylmalonyl CoA Mutase in Mice after *In Vivo* Gene Transfer with Asialoglycoprotein/Polylysine/DNA Complexes

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ABSTRACT

Methylmalonic acidemia resulting from genetic deficiency of methylmalonyl CoA mutase (MCM) is an often fatal metabolic disease. Somatic gene therapy for this disorder may require gene replacement in the liver. We describe overexpression of MCM in the liver of mice after *in vivo* gene delivery using asialoglycoprotein/polylysine/DNA (ASO/PL/DNA) targeted delivery to the liver of plasmids expressing recombinant MCM. After intravenous administration of the ASO/PL/DNA complex, the vector sequences are cleared from the blood with $t_{1/2}$ = 2.5 min and >95% of the vector is taken up by the liver. Vector sequences are cleared from the liver with $t_{1/2}$ = 1.0–1.3 hr. MCM enzyme activity in the liver increases to levels 30–40% over baseline 6–24 hr after injection. No acute or chronic toxicity was observed. This net level of expression is likely to be therapeutic for MCM if the complex could be administered repetitively to treat acute episodes of life-threatening acidosis or establish a steady-state level of MCM activity. Repetitive administration of the ASO/PL/DNA complexes in mice was associated with formation of antibodies against asialo-orosomucoid and the asialo-orosomucoid complex but not against DNA.

OVERVIEW SUMMARY

Methylmalonic acidemia is an often fatal inborn error of metabolism that might be effectively treated by repetitive administration of a gene therapy with a short duration of action such as methods described for delivery of DNA expression vectors to the liver using asialoglycoprotein/DNA complexes. The present work describes studies of the pharmacokinetics and short-term toxicology of gene delivery using these methods, demonstrating that therapeutic levels of the enzyme methylmalonyl CoA mutase can be established in the liver, and illustrating the pharmacokinetic and toxicological challenges that remain in developing clinically applicable therapies.

INTRODUCTION

METHYLMALONIC ACIDEMIA/URIA (*mut* MMA) is an often fatal inborn error of metabolism caused by deficiency of the enzyme methylmalonyl CoA mutase (MCM, EC 5.4.99.2) (Rosenberg and Fenton, 1989; Ledley, 1990a). MCM is a nuclear-encoded, mitochondrial matrix enzyme, which requires adenosylcobalamin as a cofactor (Retey, 1982; Rosenberg and Fenton, 1989). This enzyme catalyzes an intermediate step in the degradation of propionate absorbed from the gut, as well as propionyl-CoA, formed during metabolism of odd-chain fatty acids and certain branch-chain amino acids (Rosenberg and Fenton, 1989; Ledley, 1990a). While MCM is commonly considered to be a ubiquitous enzyme, the liver is the principal site

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of propionate/propionyl-CoA metabolism, as well as the organ most significantly affected by deficiency of this enzyme. The severe forms of *mut* MMA are characterized by episodic organic acidosis and a syndrome of hepatic failure with hyperammonemia, abnormalities of serum amino acids, and hypoglycemia.

Current therapy for *mut* MMA consists of dietary restriction of amino acid precursors to propionyl CoA, caloric supplementation to prevent protein catabolism, oral antibiotics to prevent fermentation to propionate, and administration of carnitine to enhance clearance of free fatty acids and prevent CoA depletion (Rosenberg and Fenton, 1989). Despite therapy, MCM is associated with >50% mortality and profound morbidity, including mental retardation, in most survivors (Matsui *et al.*, 1983; Shevell *et al.*, 1993). Liver transplantation, which should be therapeutic, has never been performed for this disease (Ledley, 1992a).

The cloning of human MCM (Ledley *et al.*, 1988; Jansen *et al.*, 1989) and the demonstration that propionate metabolism can be restored to normal levels in genetically deficient *mut* cells by DNA-mediated gene transfer (Wilkemeyer *et al.*, 1990, 1991; Andrews *et al.*, 1993) or retrovirus-mediated gene transfer (Sawada and Ledley, 1993) has introduced the potential for somatic gene therapy of *mut* MMA. Various methods and targets for somatic gene therapy have been described (Anderson, 1992; Miller, 1992; Ledley, 1993). Several observations suggest that effective gene therapy for *mut* MMA will require replacement of MCM activity in the liver (Ledley, 1990a,b; Wilkemeyer *et al.*, 1993a).

There are currently several approaches for hepatic gene therapy (Ledley, 1993). The first is an *ex vivo* strategy in which hepatocytes are harvested from a patient after partial hepatic resection, grown in culture, subjected to gene transfer using retroviral vectors that permanently integrate the therapeutic gene in the cell chromosomes, and then transplanted into the patient by hepatocellular transplantation. The feasibility of each of these steps has been demonstrated in experimental animals (Chowdhury *et al.*, 1991; Ledley *et al.*, 1993) and clinical trials of this approach for gene delivery to the liver are currently underway (Grossman *et al.*, 1994). The goal of *ex vivo* gene therapy is to place a normal MCM gene permanently in a subpopulation of hepatic cells.

A second strategy involves formulating DNA expression vectors for direct administration to patients by employing methods for targeting DNA to the liver that have been used previously for drug delivery. In particular, methods have been described for coupling DNA to an asialoglycoprotein (specifically asialo-orosomucoid, ASO), which can be administered intravenously and directs uptake of the DNA to the liver *via* the liver-specific asialoglycoprotein receptor (Wu and Wu, 1987, 1988a,b; Wu *et al.*, 1989; 1991; Wilson *et al.*, 1989, 1992). This method makes use of a complex in which ASO is covalently bound to polylysine (PL) and the negatively charged DNA is noncovalently bound to the positively charged PL. *In vivo* studies have established the validity of this approach, and have demonstrated that recombinant genes delivered using an asialoglycoprotein/polylysine/DNA (ASO/PL/DNA) complex will be expressed in the liver for several days (Wu and Wu, 1988a,b; Wu *et al.*, 1989, 1991). Such therapies could be used to treat acute episodes of *mut* MMA, which commonly last for several days, or to establish a steady-state level of the gene product by chronic administration. In fact, therapies having a finite duration of action may be preferable to permanent therapy due to the intermittent and unpredictable nature of the clinical symptoms, the tendency of the symptoms to become less severe with age, the problems inherent in life-long follow-up of patients (Ledley *et al.*, 1992), and the instances of misdiagnosis (Woolf *et al.*, 1993; Shoemaker *et al.*, 1993). Although permanent gene therapy is theoretically attractive, permanent therapy may be an unnecessary risk. Optimal clinical effectiveness and safety may require a therapy that can be administered intermittently with the dose and schedule tailored to the patient's current clinical needs or even terminated when adverse experiences are encountered. Such a therapy must be sufficiently safe to allow repetitive administration and must exhibit reproducible pharmacokinetics (Ledley and Ledley, 1993). The present work was undertaken as part of a preclinical study to assess the potential utility of ASO/PL/DNA complexes for gene therapy of *mut* MMA, to determine whether therapeutic levels of gene expression could be achieved using ASO/PL/DNA gene transfer, to describe the pharmacokinetics of vector delivery, and to explore whether repetitive administration of this complex is feasible.

MATERIALS AND METHODS

Gene transfer vectors and ASO/PL/DNA complex

The expression vector pCMV-hMCM contains the cytomegalovirus immediate early promoter, SV40 late intron, SV40 polyadenylation sequence, and the full-length human MCM cDNA (Fig. 1A). Plasmid DNA was prepared by alkaline lysis and $CsCl_2$ centrifugation (Sambrook *et al.*, 1989).

The ASO/PL/DNA complex was prepared as described (Wu and Wu, 1987). A targetable conjugate was prepared by mixing ASO with PL (poly-L-lysine, M_r = 59,000, SIGMA) in a 1:1 weight ratio in 5 ml of deionized water, and the solution was adjusted to pH 7.4. The reactants were coupled by addition of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Pierce) in a 140-fold molar excess over ASO and stirred for 16 h at 25°C. The reaction mixture was then dialyzed against deionized water at 4°C for 72 hr, lyophilized and purified by cation-exchange chromatography using a high-pressure liquid chromatographic system (Rainin) employing an Aquapore C-300 column, and stepwise elution with 0.1 *M* sodium acetate pH 5.0, 2.5, 2.25, and 2.0. The second peak eluted from the column, as detected by UV absorption at 230 nm, was determined to be the optimal conjugate form and was used for all subsequent experiments.

To form targetable complexes plasmid DNA, 0.5 mg in 1 ml of 2 *M* NaCl was added to 0.15 mg of ASO/PL conjugate in 600 ml of 2 *M* NaCl at 25°C. The mixture was then placed in 1.0-cm (flat width) dialysis tubing with an exclusion limit of 12–14,000 D (Spectrapore), and dialyzed step-wise successively at 1.5 *M*, 1.0 *M*, 0.5 *M*, 0.25 *M*, and 0.15 *M* NaCl. After the final dialysis, the complex was filtered through 0.45-μm membranes (Millipore) prior to injection. To determine the proportions of ASO–PL conjugate, the plasmid that maximized the DNA content of a soluble complex, and agarose gel retardation system was used (Wu and Wu, 1987).

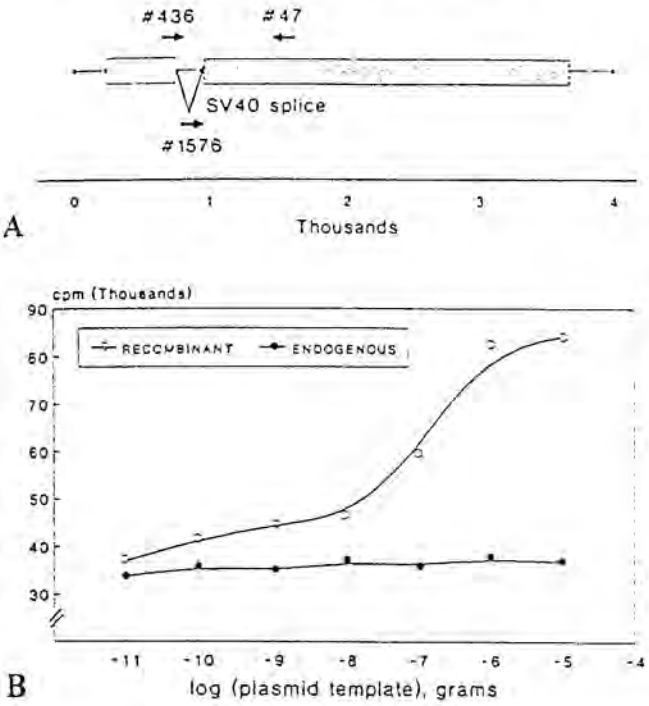

FIG. 1. Construct used for *in vivo* gene transfer (pCMV-hMCM) and strategies for semiquantitative detection of vector sequences. A. Schematic of vector showing CMV promoter (shaded box), human MCM cDNA (cross-hatched box), and SV40 intron. Oligonucleotides #436 and #47 were used to amplify vector sequences in blood and tissues. Oligonucleotides #1576 and #47 were used to amplify spliced transcripts after reverse transcription but did not amplify unmodified vector sequences. B. Sample control curve showing quantitative amplification of standard samples of vector plasmid mixed with 1 μg of murine genome DNA. Multiplex PCR was performed with oligonucleotides against the plasmid sequences (#436 and #47) and oligonucleotides against the endogenous murine MCM locus (oligonucleotides #196 and #182). There is a log linear increase in PCR product from plasmid amplification over a discrete concentration of template, and no change in amplification of genomic sequences in the multiplex reaction.

Animal procedures

ASO/PL/DNA complexes containing 100 μg of plasmid DNA in 300 μl of sterile saline was injected into the tail vein of 10- to 12-week-old ICR female mice. Several animals received four injections of the complex over 8 months. All experiments were performed in duplicate animals.

Blood was collected from the tail and serum was separated for polymerase chain reaction (PCR) analysis. Organs were harvested after Nembutal euthanasia. To assess the cellular content of vector DNA and eliminate potential contaminations from DNA in the vascular space, animals were perfused by intracardiac injection of 5% dextrose in 75 m*M* NaCl prior to removal of the organs. Perfusion was performed through a midline incision by catheterization of the left heart with a 20-gauge catheter and transection of the inferior vena cava until the eluate from the vena cava was clear.

Recombinant DNA procedures

Total DNA was prepared by SDS-proteinase K digestion, phenol/chloroform/isoamylalcohol extraction, and ethanol precipitation. Total RNA was isolated from tissues with RNAsol (CINNA/BIOTECX). Other recombinant procedures were performed using standard methods (Berger and Kimmel, 1987; Sambrook *et al.*, 1989).

Vector DNA was identified in serum or in DNA purified from tissues by semiquantitative PCR using oligonucleotides complementary to the cytomegalovirus (CMV) vector and human MCM sequences (oligonucleotides #436 and #47; Fig. 1A). PCR was performed using 2 μl of serum or 1 μg of purified DNA in a 50-μl volume with 5 pmol primers and standard reaction conditions (Berger and Kimmel, 1987). The reaction was performed by denaturation at 94°C/5 min and 21–35 cycles of PCR at 92°C/1 min, 61°C/1.5 min, 74°C/1 min with a final extension at 74°C/3 min. All solutions were treated with UV light to minimize contamination (Ou *et al.*, 1991). All PCR reactions were run with negative controls, including samples from untreated animals harvested simultaneously and reaction blanks containing all reagents in the absence of template.

A semiquantitative standard was run contemporaneously with each experiment by amplification of standards containing normal mouse DNA or mouse serum mixed with 10^{-9} to 10^{-15} grams of pCMV-hMCM plasmid DNA. Reaction products were visualized by gel electrophoresis and bidirectional Southern blotting. Quantitation was performed using a Betascope 603 Blot Analyzer (Betagen Corp., Waltham, MA). Control experiments demonstrate that the amount of reaction product is related to the amount of plasmid template in a log-linear fashion after 21–25 cycles of PCR. A sample control curve is shown in Fig. 1B. As an internal control, a multiplex reaction was run with two additional oligonucleotides that amplify a portion of the endogenous murine *mut* locus (Wilkemeyer *et al.*, 1993b). This control demonstrates that there is no significant change in the amplification of the endogenous sequences between samples (Fig. 1B). Increasing the number of PCR cycles provided greater sensitivity, but the quantitative relationship between the amount of template and the amount of product is lost over this range.

Oligonucleotide sequences: #436, 5′-TGACCTCCATAGAAGACACCGGGAC; #47, 5′-GCATACTGGCGGATGGTCCAG; #182, 5′-ACAGTGTTTGGGGATCACCCG; #196, 5′-ATTTGATTGGCTGCCGAGCTC; #1576, 5′-AACCAGAAAGTTAACTGGCCT. Other oligonucleotide sequences are given in Wilkemeyer et al. (1993b).

The half-life ($t_{1/2}$) of vector sequences (S) as a function of time (t) was calculated from the rate constant for elimination (k_e) where: $k_e = -\log[S]/t \times 2.3$ and $t_{1/2} = 0.693/k_e$ (Gibaldi and Perrier, 1982).

MCM mRNA expression was identified by coupled reverse transcription (RT)-PCR. For this reaction 25 μg of total RNA was digested with RNase-free DNase and reverse-transcribed from random hexamer primers (PHARMACIA/LKB) with avian reverse transcriptase (Berger and Kimmel, 1987). An aliquot comprising one-fifth of this reaction was used as template for PCR using a 5′ oligonucleotide which corresponds to the spliced mRNA. This oligonucleotide spans the 16S splice site from the SV40 intron with only three bases 3′ to the splice

acceptor site (Fig. 1A). Control experiments demonstrated that these oligonucleotides would not amplify unspliced sequences. Control reactions included non-reverse-transcribed RNA, nontransduced samples, and reaction components in the absence of template. This is a nonquantitative assay that is intended to confirm the expression of mRNA conjunction with the expression of MCM enzyme activity. Because this assay is nonquantitative, the presence of RNA serves essentially as a control to support the observation of increased enzyme in the livers of injected animals.

Enzymatic and immunological assays

MCM enzyme activity was assayed as the conversion of [^{14}C]methylmalonyl CoA to [^{14}C]succinate as described (Kolhouse *et al.*, 1988; Andrews *et al.*, 1993). Protein was determined using the Bradford microassay (BioRad). All assays were performed with the addition of excess adenosylcobalamin (66 μM) to measure total enzyme, as opposed to only holoenzyme. Results are expressed as nmol succinate formed/mg protein per hour calculated as the mean of triplicate samples. Standard deviations were <5% of the mean.

Antibodies against the ASO/PL/DNA complex were assayed by immunoprecipitation of [^{125}I]ASO/PL/DNA complexes, [^{125}I]ASO, or [^{32}P]DNA. [^{125}I]ASO was prepared using Bolton Hunter reagent and [^{125}I]ASO/PL/DNA complex was assembled using [^{125}I]ASO as described (Wu and Wu, 1987). Precipitations were performed with [^{125}I]ASO/PL/DNA containing 25 ng of ASO, 5 ng of PL, and 0.1 ng of DNA. [^{32}P]DNA was prepared using random hexamer primers. Immunoprecipitation of labeled substrates by preimmune mouse serum as well as serum from animals injected four times with the ASO/PL/DNA complex was assayed as described (Firestone and Winguth, 1990) using fixed staphylococcus A cells (Pansorbin, CalBiochem). Competition experiments were performed with the addition of 10- to 100-fold molar excess of unlabeled polylysine, orosomucoid, ASO, plasmid DNA, or combinations of these materials in the same ratio in the ASO/PL/DNA complex. All precipitation experiments were performed in duplicate with variation <10% of the mean. Anti-nuclear antibody (ANA) was assayed by the clinical pathology laboratory of Texas Children's Hospital using goat anti-mouse IgG.

RESULTS

Clearance and targeting of vector sequences from ASO/PL/DNA complex after intravenous injection

Semiquantitative PCR was used to assay for the presence of vector sequences in serum 1, 5, 10, 30, 60, and 120 min after injection of ASO/PL/DNA containing 100 μg of vector (Fig. 2). Vector sequences were evident in serum drawn immediately after injection and levels decreased rapidly. First-order kinetics were apparent from the log-linear relationship between time and the log of the concentration of vector sequence ($R^2 = 0.98$), with the half-life calculated to be $t_{1/2} = 2.5$ min. No vector was detectable by up to 36 cycles of PCR after 30 min.

The distribution of vector sequences was assessed 1 hr after injection. The highest concentrations were evident in liver, with lower concentrations in spleen and lung and little in other tissues (Fig. 3). Semiquantitative analysis indicated that >98% of the injected vector was taken up by the liver which is consistent with previous data (Wu and Wu, 1988a) (Fig. 3).

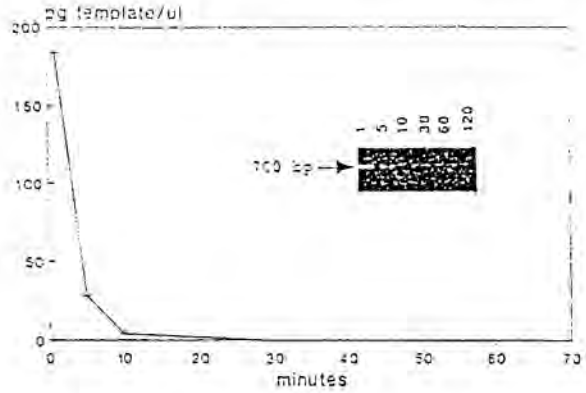

FIG. 2. Clearance of vector sequences from serum. Vector sequences were identified in serum drawn at intervals after administration of the ASO/PL/DNA complex. The concentration of vector sequences was determined by comparison with a semiquantitative control curve. Results are expressed in pg of template/μl of serum. Insert, Ethidium bromide-stained gel showing amplification products.

Degradation and persistence of vector sequences in the liver

The concentration of vector sequences in the liver was determined by semiquantitative PCR using DNA purified from tissue harvested at different times after injection (Fig. 4). The highest concentrations were observed at the earliest time point taken (1 hr) with approximately 10^6 copies of the PCR template/cell. First-order kinetics were apparent during the first 24 hr after

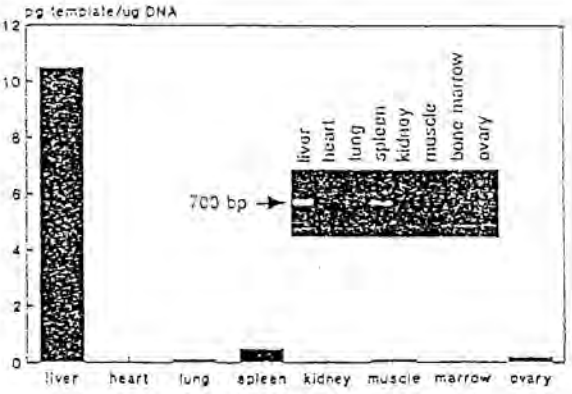

FIG. 3. Tissue-specific uptake of vector sequences from ASO/PL/DNA complex. Vector sequences were identified in DNA purified from different tissues 1 hr after administration of the ASO/PL/DNA complex. The concentration of vector sequences in different tissues was determined by comparison with a semiquantitative control curve. Results are expressed in pg of template/μg of total DNA. Insert, Ethidium bromide-stained gel showing amplification products.

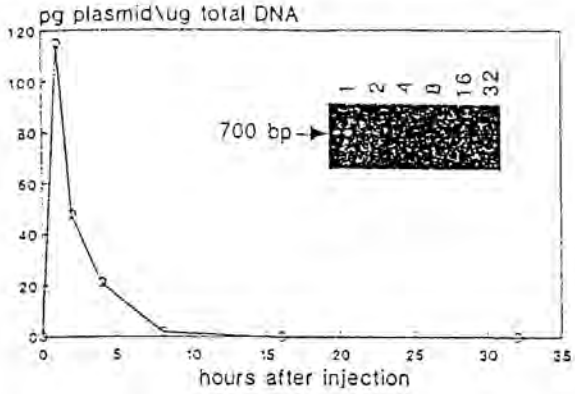

FIG. 4. Clearance of vector sequences from liver. Vector sequences were identified in DNA purified from liver of animals sacrificed at intervals after administration of the ASO/PL/DNA complex. The concentration of vector sequences determined by comparison with a semiquantitative control curve. Results are expressed in pg of template/μg of total DNA. Insert, Ethidium bromide-stained gel showing amplification products.

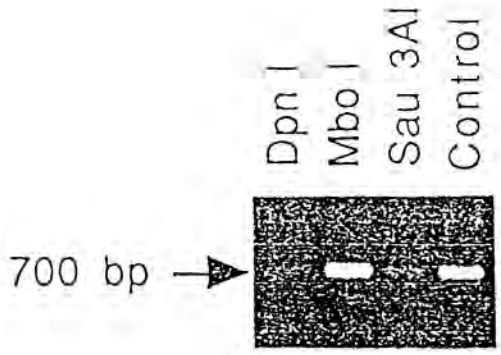

FIG. 5. Pattern of DNA methylation in material amplified from tissues 30 days after administration of the ASO/PL/DNA complex. Vector sequences were detectable by 36 cycles of PCR as late as 30 days after injection. The pattern of methylation was assessed by digestion of total DNA with methylation-sensitive enzymes *Dpn* I or *Mbo* I, or the methylation-insensitive enzyme *Sau* 3A prior to PCR. A decrease in PCR intensity reflects sensitivity of the template to the enzyme. Sequences that are not replicated would retain the bacterial pattern of methylation (*Dpn* I and *Sau* 3A sensitive, *Mbo* I resistant).

injection by the log-linear relationship between time and the log of the concentration of vector sequences (R^2 = 0.78–0.92) with a calculated half life $t_{1/2}$ = 1.0–1.3 hr.

Vector sequences were never completely eliminated from the liver. In samples taken as late as 30 days after injection (the last time point examined), vector sequences could be amplified from the livers of all animals using 35 cycles of PCR at levels representing approximately <1 copy/10^2–10^3 cells. To determine whether these sequences were replicated, we assayed for the bacterial pattern of methylation (*Dpn* I-sensitive/*Mbo* I-resistant) or the mammalian pattern of methylation (*Mbo* I-sensitive/*Dpn* I-resistant) by digesting total DNA with these enzymes prior to PCR amplification (Fig. 5). *Sau* 3A, which is methylation independent, was used as a control to demonstrate the decrease in PCR amplification after digestion. The amplification signal was found to be sensitive to *Sau* 3A and *Dpn* I but not *Mbo* I, suggesting that these sequences retained the bacterial pattern of DNA methylation.

MCM expression in the liver

MCM enzyme assays were performed on soluble protein from the livers of treated animals and controls (Fig. 6). MCM activity was increased in the livers of animals sacrificed 6–24 hr after injection. Animals sacrificed more than 48 hr after injection exhibited levels of enzyme activity indistinguishable from normal. The level of gene expression 24–48 hr after injection averaged 30–40% higher than normal.

We used a nonquantitative RT-PCR assay to identify mRNA transcripts from the pCMV vector in the liver after gene delivery. Control experiments demonstrate that the PCR strategy used is specific for transcripts from the recombinant gene and will not amplify either the plasmid or the endogenous MCM transcripts (Fig. 7A). Using this assay, we identified mRNA transcripts originating from the pCMV-hMCM vector that were evident in the liver 6–24 hr after injection using a nonquantitative, RT-PCR assay. This analysis made use of an oligonucleotide primer that recognized the spliced transcripts but not plasmid DNA, the endogenous MCM gene, or reverse-transcribed endogenous murine mRNA (Fig. 7B). No signal was apparent at 48 hr or beyond or in controls that included samples from noninjected animals and RNA from treated animals subjected to PCR without reverse transcription. The presence of amplifiable mRNA is consistent with the increased levels of enzyme activity identified in the livers of injected animals and confirms the expression of recombinant MCM after *in vivo* gene delivery.

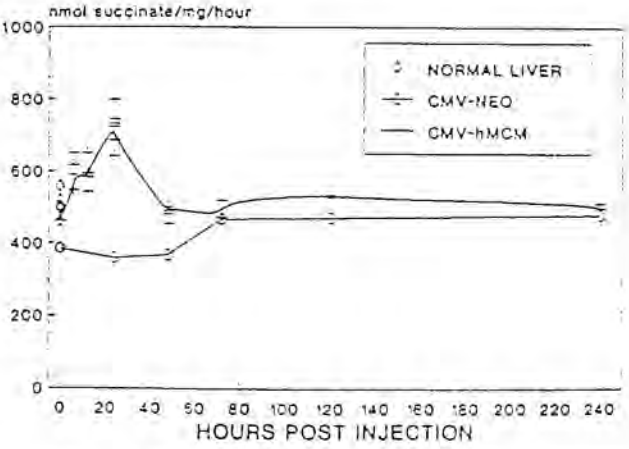

FIG. 6. MCM enzyme activity in the liver after administration of the ASO/PL/DNA complex. Animals were sacrificed at intervals and MCM assayed in total protein extracts. Significant elevations of enzyme activity were apparent between 6 and 24 hr after administration in animals treated with the CMV-hMCM complex but not CMV-NEO-R control. After 48 hr, the level of MCM activity was equivalent to baseline.

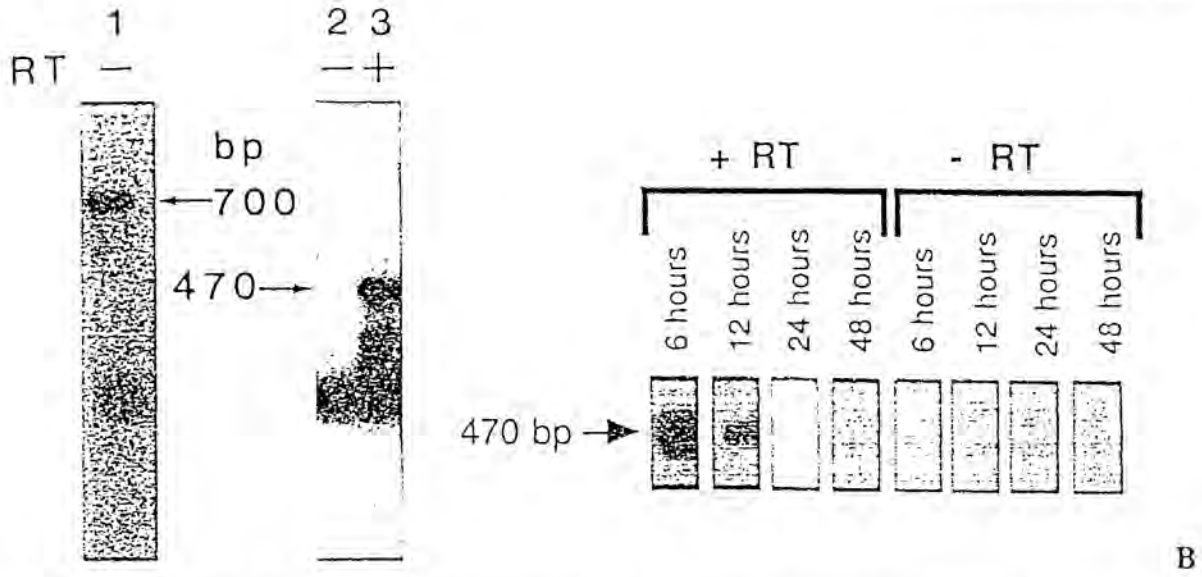

FIG. 7. Detection of vector transcripts by reverse transcription PCR. Transcripts arising from the expression vector were identified using an oligonucleotide that spans the SV40 16S intron and amplified only spliced sequences. A. Control experiments demonstrate that this PCR reaction will amplify transcripts arising from the pCMV vector, but not plasmid DNA or the endogenous murine MCM transcripts, is shown for a sample experiment. Lane 1, Amplification of pCMV-hMCM plasmid in purified "RNA" using oligonucleotides #436 with 47 present in plasmid sequence; lane 2, absence of amplification in non-reverse-transcribed material using junction oligonucleotide #1576 with #47; lane 3, specific amplification in reverse-transcribed material using junction oligonucleotide #1576 with #47. B. Transcripts arising from the pCMV-MCM vector were identified in RNA purified form liver tissue at different times after administration of the ASO/PL/DNA complex. No amplification is apparent in control samples including RNA amplified without prior reverse transcription.

Toxicities of ASO/PL/DNA delivery

No acute toxicities were observed after a single administration of the ASO/PL/DNA complex. To investigate the feasibility of repetitive administration of ASO/PL/DNA complexes, 4 mice were injected at four different times over a period of 8 months with the ASO/PL/DNA complex. No chronic toxicities were observed in animals maintained for 9 months after injection. One animal, however, died within 10 min after the fourth injection with signs of cyanosis and respiratory distress. This was the only animal to die after injection among >100. Autopsy on this animal showed no gross abnormalities and nonspecific pulmonary congestion consistent with, but not pathognomonic of, anaphylaxis.

To investigate whether antibodies had been formed against the ASO/PL/DNA complex, the remaining 3 animals were sacrificed 10 days after the last injection when antibody titers were expected to be maximal. Complete autopsies on these animals were performed and were unremarkable. Serum from all 3 animals was assayed for antibodies against the ASO/PL/DNA complex. All three antisera precipitated [^{125}I]ASO/PL/DNA complexes (Fig. 8A) at dilutions of >1:1,000 (Fig. 8B). The specificity of the antisera was assessed by competition with cold substrates at concentrations 10- and 100-fold higher than in the [^{125}I]ASO/PL/DNA complex. Precipitation of [^{125}I]ASO/PL/DNA was not inhibited by unlabeled DNA, was partially inhibited by unlabeled PL, and was completely inhibited by unlabeled ASO or by a mixture of ASO + PL + DNA at ratios similar to those in the ASO/PL/DNA complex (Fig. 8C). These results suggest that antibodies were formed against both the ASO and PL components of the covalent ASO/PL complex but not the noncovalently bound DNA. Two studies were performed to rule out the presence of antibodies against DNA. First, antiserum from injected animals did not precipitate [^{32}P]DNA more than preimmune serum. The small number of counts present in the precipitate (<0.1% of total counts) was equivalent with immune and preimmune serum and was not altered by the addition of 10- to 100-fold excess unlabeled DNA (Fig. 8D). In addition, antinuclear antibodies (ANA) were assayed using conventional clinical methods adapted to identify murine antibodies. This test for ANA was negative (data not shown).

DISCUSSION

The clinical characteristics of *mut* MMA make this disease an attractive candidate for somatic gene therapy: (i) it can be diagnosed before the onset of irreversible disease by prenatal diagnosis (Ampola *et al.*, 1975) or newborn screening (Coulombe *et al.*, 1981; Ledley *et al.*, 1984); (ii) it is associated with serious morbidity and mortality despite conventional therapy (Matsui *et al.*, 1983; Shevell *et al.*, 1993); and (iii) *in vitro* studies have demonstrated that MCM deficiency can be corrected in genetically defective *mut* fibroblasts by DNA or retrovirus-mediated gene transfer. The clinical and biochemical characteristics of the *mut* MMA suggest that the liver represents the preferred target for somatic gene therapy (Wilkemeyer *et al.*, 1991; Sawada and Ledley 1993; Wilkemeyer *et al.*, 1993a).

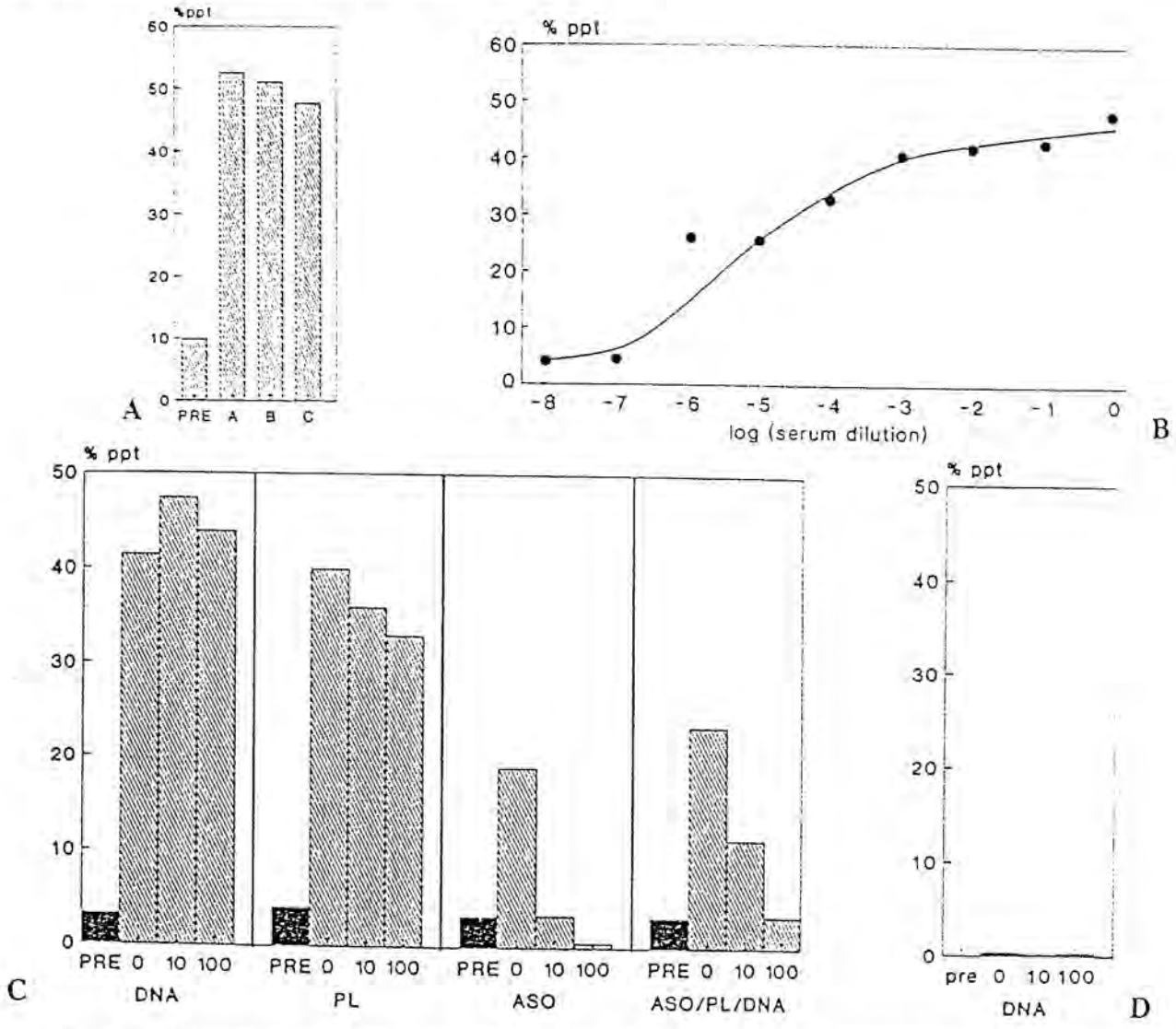

FIG. 8. Characterization of antibodies formed against the ASO/PL/DNA complex. A. Precipitation of [^{125}I]ASO/PL/DNA complex by serum from three animals (A, B, and C) who received four successive injections of complex over a period of 8 months. Serum was collected from each animal 10 days after the last injection. Preimmune serum (PRE) is from a littermate who was not injected with complex. The experiment was performed with a 1:1,000 dilution of mouse serum. B. Titer of antibody against [^{125}I]ASO/PL/DNA complex. C. Competitive inhibition of [^{125}I]ASO/PL/DNA precipitation by DNA, PL, ASO, and a mixture of ASO + PL + DNA. Competition was performed using a 1:1,000 dilution of serum and unlabeled materials at molar concentrations 10- and 100-fold higher than their composition in the ^{125}I-labeled material. The ASO + PL + DNA mixture contained molar ratios of each component equivalent to those in the ASO/PL/DNA complex and was used at concentrations 10- and 100-fold higher than the [^{125}I]ASO/PL/DNA target. D. Absence of precipitation of [^{32}P]DNA with a 1:1,000 dilution of mouse serum. Precipitation represents <0.1% of [^{32}P]DNA added to the reaction with no significant difference between counts precipitated by preimmune serum, immune serum, or immune serum plus excess unlabeled DNA.

The intent of the present studies was to evaluate whether current methods for delivery of DNA expression vectors to the liver could be suitable for therapy of *mut* MMA. The premise of this approach is that a DNA vector that produces a potent effect of finite duration, might be efficacious in managing *mut* MMA if the gene could be administered repetitively like a conventional medicine. The clinical characteristics of *mut* MMA may make this disorder particularly appropriate for such therapy. Even the most severe forms of *mut* MMA are characterized by long periods of stability on conventional dietary therapy punctuated by episodes of acute, life-threatening acidosis triggered by infection or noncompliance with therapeutic regimens which last several days. Although some patients have numerous hospitalizations for acidosis during the first years of life (Rosenberg and Fenton, 1989), others have only rare episodes (Shapira *et al.*, 1991). Moreover, it has been observed that children who survive through the first 5–6 years of life may be less prone to acute episodes of dyshomeostasis, even though they remain MCM deficient and continue to exhibit organic acidemia or aciduria. Thus, the clinical effectiveness of gene

therapy could be enhanced if the physician has the ability to alter the dose or schedule of administration to match the patient's immediate clinical needs. Finally, as evidenced by recent, well-publicized cases of misdiagnosis for MMA (Shoemaker *et al.*, 1993; Woolf *et al.*, 1993), misdiagnosis will occur for genetic diseases, as for common diseases. Moreover, adverse experiences may be reported for various reasons related to the administration of the gene therapy, or other unforseen events. Thus, the ability to terminate therapy could provide an important margin of safety in clinical practice.

The present studies were undertaken to assess the feasibility of this approach using methods for gene delivery to the liver with ASO/PL complexes described previously. In previous studies performed using the same conjugate system, prepared in the same manner, and using plasmids of similar size, uptake of radiolabeled complex as determined by autoradiography revealed that >90% of the grains were located over hepatocytes (Chowdhury *et al.*, 1993). Approximately 5–10% of grains were found to be present over Kupffer cells, and the proportion of grains/cell was equal for hepatocytes and Kupffer cells. In further experiments, complexed DNA was recently injected intravenously into groups of rats and their livers were perfused to separate hepatocytes from nonparenchymal cells. Foreign DNA was identified and quantitated by Southern blots. These studies demonstrated that plasmid was taken up by hepatocytes at a level approximately 20,000 copies/cell, and accounted for approximately 95% of the injected DNA. This DNA extracted from hepatocytes was capable of being retransfected into bacteria, resulting in the generation of new DNA of the same size and restriction pattern as the original plasmid. Nonparenchymal cells took up approximately 5% of DNA (A.W. Wu and C.H. Wu, unpublished data). Together, these data indicate that the vast majority of the complexed DNA is taken up by hepatocytes. These data are consistent with the expectations from experiments with targeting conventional drugs to the liver using asialoglycoproteins (Chowdhury *et al.*, 1993).

The present studies confirm previous reports (Wu and Wu, 1987, 1988a,b; Wilson *et al.*, 1989, 1992; Wu *et al.*, 1989, 1991) that the injected complex is rapidly cleared from the blood, that the complex is preferentially taken up by the liver, and that vector sequences are largely cleared from the liver within 24 hr of administration. Our analysis differs from previous studies in that we used PCR to detect the vector sequences in blood and tissues rather than radioisotopes affixed to the protein or DNA. We chose to use PCR, which reflects not only the distribution and disposition of the ASO/PL/DNA complex and its components, but also the approximate concentration of molecules that might serve as templates for transcription. Specifically, PCR will not detect DNA catabolized in the blood or tissues.

Our studies demonstrate that MCM activity increases to levels as high as 30–40% over the normal levels in murine liver approximately 24 hr after administration. We have previously shown that murine MCM exhibits kinetic properties similar to human MCM and is expressed in the liver at similar levels (Wilkemeyer *et al.*, 1990). Thus, the increase in MCM activity observed in these experiments corresponds to expression at levels up to 30–40% of normal human liver. This is significantly above the threshold that would be considered "therapeutic." We have observed, for example, that patients having a mutation that preserves as little as 1% normal activity exhibit an intermediate phenotype of *mut* MMA (Crane *et al.*, 1992).

We observed a significantly shorter duration of expression of MCM than described previously and a shorter than expected half-life for DNA in the liver. In a separate study we have constructed a numerical, computer model of the pharmacokinetics of gene delivery to cells to understand how the intrinsic half-life of the DNA, RNA, and gene product, the rates of transcription and translation, and the kinetics of flux between various intracellular compartments affects the apparent kinetics of the gene product (Ledley and Ledley, 1994). These studies demonstrate that despite the multiplicity and complexity of the intrinsic kinetic processes that may affect the apparent level and rate of elimination of the gene product, these kinetic parameters will be predominantly determined by the "rate-limiting" clearance of the DNA, RNA, or gene product, whichever exhibits the longer $t_{1/2}$. Thus, while DNA may have a short $t_{1/2}$, prolonged clearance of the product can reflect a longer $t_{1/2}$ of the mRNA or gene product. The present data may be explained by the fact that MCM may have a significantly shorter half-life than proteins such as CAT or albumin that have been studied previously.

The therapeutic potential of repetitive administration of DNA-vectors using the ASO/PL/DNA complex may be limited by the antibody response observed in response to the covalently modified ASO. It should be emphasized that no antibodies were detected against DNA by direct immunoprecipitation, competition, or antinuclear antibody tests. This is important, because antibodies against DNA might preclude this approach to therapy. Further studies will be needed to assess the functional significance of the immunological response to ASO/PL, whether these antibodies block the activity of the ASO/PL/DNA complex, or whether these antibodies will alter the pharmacokinetics of such therapy. It should be noted that many biological products are associated with seroconversion in clinical use, although such antibodies can complicate the dose response and can cause immunological reactions.

Our data also demonstrate that small amounts of DNA, detectable only by PCR, are evident in the liver as long as 30 days after administration of the ASO/PL/DNA complex. This DNA apparently retains the bacterial pattern of methylation, suggesting that it has not been replicated or integrated into the genome. The persistence of the bacterial pattern of methylation has also been observed when subtotal hepatectomy is performed in conjunction with ASO/PL/DNA-mediated gene transfer to enhance the duration of recombinant gene expression (Wilson *et al.*, 1992). The safety of ASO/PL/DNA complexes would be enhanced if this DNA were completely eliminated from the target cells after administration.

In conclusion, these studies in mice demonstrate the feasibility of constituting short-term MCM expression in the liver at potentially therapeutic levels using ASO/PL/DNA gene transfer. There may be significant advantages to such therapies that may be administered by conventional routes and may embody a higher margin of effectiveness and safety than permanent therapies.

These studies highlight several issues that remain to be addressed in developing efficacious products for somatic gene therapy, particularly the need for further characterization of the toxicology of the gene and gene product. *Mut* MMA may be a

useful model for further studies, one in which clinical trials of transient gene therapy for acute exacerbations of acidosis may be justified if repetitive administration proves to be feasible and safe.

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IV.számú közlemény

Génterápia – valóság vagy fantázia

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Összefoglalás

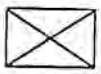
A molekuláris genetika előrehaladásával mintegy évtizedes vita lezárásaként 1989-ben megkezdődtek az első szomatikus génterápiás klinikai kísérletek. Az alábbiakban áttekintésre kerülnek a génterápia alapvető módszerei és azok klinikai alkalmazásának elméleti vagy gyakorlati lehetőségei. Bár minden bizonnyal jelentős előrehaladás várható a jövőben a génterápia terén, a ma meglévő eljárások is figyelemre méltó lehetőséget nyújtanak a klinikai alkalmazást illetően.

Kulcsszavak:
génterápia, vírus mediálta géntranszfer,
DNS mediálta géntranszfer,
retrovírus vektorok

A molekuláris genetika elmúlt évtizedben történt fejlődése megteremtette egy új eljárás, a génterápia alkalmazásának lehetőségét olyan genetikai vagy szerzett megbetegedésekben, melyek a hagyományos kezelési eljárásokkal nem, vagy csak inadekvát módon kezelhetők. Annak ellenére, hogy világszerte a gyakorló orvosok nagy része a génterápiát csupán a távoli jövő lehetőségének tartja, fontos felkészülni a majdani orvosi gyakorlatban történő alkalmazásra. A nyolcvanas években a szomatikus génterápia klinikai alkalmazása még pusztán elméleti lehetőségnek tűnt, és sok nehéz kérdés, mint a technikai kivitelezhetőség, a biztonság, az emberen végzett génmanipuláció szociális és etikai vonatkozásai megválaszolatlanok voltak. Az elmúlt évtized mindezen területeken gyors fejlődést hozott, új technikai eljárásokat dolgoztak ki, részletes vizsgálatokat végeztek az eljárások biztonságosságának megítélésére. Ezek az eredmények vezettek az első klinikai alkalmazáshoz 1989-ben.[20] Azóta több mint száz azoknak a betegeknek a száma, akiknél valamilyen formában génterápiás eljárást alkalmaztak. A klinikai kísérletek során elkezdődött a szomatikus génterápia alkalmazhatóságának részletes vizsgálata, illetve azon betegségcsoportok és betegek pontos meghatározása, ahol a génterápiás eljárások a siker reményében alkalmazhatók. Napjainkban a génterápiát már nem tekintjük pusztán a molekuláris és sejtbiológia egyszerű alkalmazásának, hanem olyan komplex kezelési eljárásnak, melynek farmakológiai, klinikai, sebészeti és ápolási aspektusait egyaránt figyelembe kell venni az esetleges gyógyászati alkalmazásban.

Miért éppen génterápia?

A szomatikus génterápia alapelve, hogy rekombináns gének *testi sejtekbe* történő bejuttatásával lehetővé válik genetikailag károsodott funkciók helyreállítása, vagy bizonyos betegségek lefolyásának megvál-



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toztatása. A génterápia alkalmazása legelőször olyan örökletes megbetegedésekben merült fel, melyek kezelése jelenlegi módszereinkkel nem megoldott. Ilyen klasszikus megbetegedés többek között a haemophilia, thalassemia, vagy a cysticus fibrosis, melyeknél a hiányzó génproduktum pótolható lenne a szomatikus génterápia segítségével.

A pathomechanizmus megértésével egyre inkább felismerésre kerül, hogy a génátvitel alkalmas lehet a betegségek sokkal szélesebb skálájának kezelésére is, ideértve a multifaktoriális megbetegedéseket, pl.: degeneratív kórképek (Parkinson-kór), hypercholesterinaemia, de akár autoimmun és gyulladásos betegségeket is azáltal, hogy azok pathophysiológiai lefolyását a génmanipuláció megváltoztatja.

Miért vannak a génterápiával kapcsolatban aggodalmak?

Különböző kérdések merültek fel a génterápiával kapcsolatban, melyek közül a legalapvetőbb az ivarsejtek manipulációjával kapcsolatos.[7, 27] Ez ugyanis felveti a következő generációk génállományába történő beavatkozás, illetve a géntechnológia esetleges eugenicus célokra történő kihasználásának lehetőségét.

Külön problémák merültek fel a kísérleti eljárások biztonságosságával, a klinikai kísérletek megtervezésének etikai vonatkozásaival és az eljárásoknak a társadalomra rótt anyagi terheivel kapcsolatban.

Azokban az országokban, ahol a génterápia, illetve génmanipuláció törvényileg szabályozott, a jogszabályok kimondják, hogy klinikai kísérletek során géneket csak és kizárólag testi sejtekbe lehet bejuttatni. Így a szomatikus génterápia alapelvét tekintve nem különbözik a szövet, illetve szervtranszplantációtól vagy protetikus anyagok beültetésétől.

A szomatikus génterápia általános alapelvei:

A szomatikus génterápiában alkalmazott eljárások elkülöníthetők aszerint, hogy milyen módszereket alkalmaznak a rekombináns gének testi sejtekbe történő bejuttatásához, mi a génátvitelre kiválasztott célszerv, mennyi a rekombináns gén sejten belüli persistálásának időtartama, illetve milyen a létrehozott génexpresszió hatásfoka.

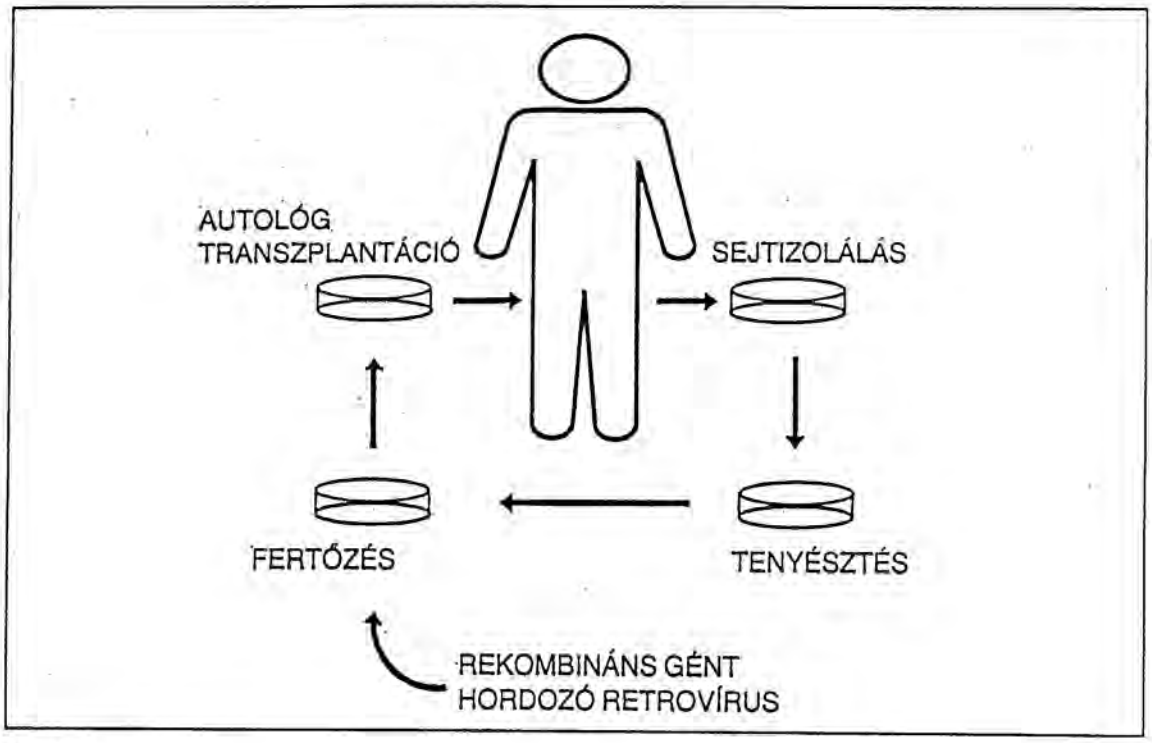
A gének sejtekbe történő bejuttatására két fő módszer ismert: I. A *vírus mediálta géntranszfer,* mely eljárás magába foglalja víruspartikulumok használatát a gének sejtekbe juttatásának céljából. Ezt az eljárást nevezzük általánosságban transdukciónak. II. A *DNS mediálta géntranszfer,* amely alatt a tisztított, vagy valamilyen hordozómolekulához kötött DNS sejtekbe juttatását értjük. Ezt az eljárást nevezzük transfectiónak.

I. Vírus mediálta géntranszfer:

1. Ex vivo szomatikus génterápia:

A génterápiának ez a ma már klasszikusnak mondható módszere alapjait tekintve magába foglalja a betegből biopsia vagy sebészi rezekció útján nyert szövetmintákból történő sejtizolálást, majd a sejtkultúrában történő tenyésztést, a sejtek rekombináns géneket tartalmazó víruspartikulumokkal való in vitro fertőzését, végül a betegbe történő visszajuttatását autológ transzplantáció révén.[16] *(1. ábra)*

Ennek az eljárásnak az a célja, hogy az izolált sejtekbe végleges (permanens) módon bejuttassuk azokat a géneket, melyek a beültetést és megtapadást követően biztosítani tudják a terápiás effektust. Ezt a megközelítést első ízben csontvelői sejtek manipulációjánál javasolták, mivel a csontvelő nyerése, tenyésztése illetve transzplantációja már relatíve egyszerűnek és biztonságosnak volt tekinthető.[19] Később számos más sejtféleség került szóba, mint lehetséges célpont, lymphocyta,[20] fibroblast,[2, 5, 12] hepatocyta,[1, 14, 5] myoblast,[23] pajzsmirigy acinussejt,[9] epidermális sejt,[17] endothel sejt,[18, 28, 34] synovialis sejt, központi idegrendszeri sejt, sőt még tumorsejtek is. Az ex vivo géntranszfer legáltalánosabb eszköze az egér leukémiavírusból származó rekombináns retrovírus. A leukémia vírusból génmanipulációs eszközökkel létrehozhatók úgynevezett de-



1. ábra

Ex vivo retrovírus mediálta génuranszfer lépéseinek sematikus vázlata

fektív törzsek, melyek képesek a rekombináns géneket a célsejtekbe bejuttatni, illetve ezen géneket a gazdasejt kromoszómaállományába permanens módon integrálni anélkül, hogy ezek a vírustörzsek bármiféle pathológiai funkcióval rendelkező virális gént hordoznának. Az egyetlen teoretikus veszélye ezen vektorok alkalmazásának az inserciós mutagenezis illetve a természetes rekombináció során fellépő malignitás kialakulása. Az ilyen irányban végzett kiterjedt laboratóriumi és klinikai kísérletek azonban semmi ilyen nem kívánt hatást nem tudtak kimutatni. Jóval reálisabbak a gyakorlati alkalmazás során felmerülő technikai problémák: nem megoldott a víruspartikulumok nagy mennyiségben történő előállítása, hiányzanak a klinikai tapasztalatok – a csontvelői valamint az epidermális sejtek transzplantációját kivéve – a célsejtek manipulációjával kapcsolatban. A legnagyobb gondot azonban a gének reimplantációt követő hosszú távú expressziója jelenti, ugyanis ez ideig nem sikerült néhány hétnél vagy hónapnál tovább tartó in vivo génexpressiót elérni.

Az ex vivo vírusközvetítette génátvitel gyakorlatban történő alkalmazására több klinikai kísérlet folyik, pl. *Economu és mtsa*[10] tumor necrosis faktor génjét ültették tumor infiltráló lymphocytákba, feltételezve, hogy a tumorba bejutva ezek e sejtek képesek lesznek tumorölő hatást kifejteni. *Blaese és mtsai*[3] SCID (sever combined immune deficiency) beteg perifériás véréből izolált lymphocytákba juttatták be az adenosin deaminase gént, majd a sejtek visszaültetése után jelentős klinikai javulást észleltek betegükben. Több protokollt dolgoztak ki relapsusban lévő leukémiás betegek csontvelősejtjeibe történő géntranszfer terápiás lehetőségeinek tanulmányozására.[4,7,8]

Jelenleg két kísérletsorozat is folyik májsejtekbe történő génbevitel tanulmányozására.

Az egyikben markergének segítségével vizsgálják a májsejt-transzplantáció technikai kivitelezhetőségét,[14] a másikban familiaris hypercholesterinaemiában szenvedő betegek májsejtjeibe juttatták az emberi LDL receptor génjét.[29]

2. In vivo szomatikus génterápia:

Vírusvektorokkal létrehozott géntranszfer másik lehetősége a rekombináns géneket tartalmazó vektorok szövetekbe történő direkt bejuttatása, mely feleslegessé tenné a technikailag nehézkes sejtizolálás, sejttenyésztés és transzplantáció lépéseit. Az ex vivo génátvitel céljaira kifejlesztett rekombináns retrovírusok azonban nem bizonyultak megfelelőnek in vivo, mivel ezek a humán complement hatására a szervezetbe történő direkt bejuttatást követően gyorsan inaktiválódnak. Jelenleg jó néhány olyan új vírustörzs van kifejlesztés alatt, melyek képesek in vivo génátvitel illetve génexpresszió létrehozására.

Adeno- illetve adeno-asszociált vírus, ugyanúgy, mint a retrovírusok, defektívvé, azaz szaporodásra és sejtlysisre képtelenné tehetők, megtartva permanens génintegrációs képességüket. Potenciális előnyük ezeknek a törzseknek, hogy beépülésük a genom egy bizonyos meghatározott régiójában történik, csökkentve ezáltal az insertiós mutagenezis lehetőségét, másrészt a génexpresszió hatásfoka nagyságrendileg jobb a retroviralis vektorokhoz képest.

Nemrégiben jelentek meg az első kísérleti eredmények, melyekben eredményesen juttattak be géneket kísérleti állatok tüdejébe illetve májába adenovírusvektorok segítségével.[22, 25]

A kezdeti lelkesedést azonban lehűtötték azok az adatok, melyek az adenovírus vektorok in vivo antigenikus illetve gyulladáskeltő hatásairól számolnak be.

Herpeszvírusvektorok alkalmazására azon tulajdonságuk keltette fel a figyelmet, hogy képesek a központi idegrendszerben hosszú ideig látens módon tartózkodni. Így alkalmasak lehetnének a hosszú távú génexpresszió fenntartására anélkül, hogy közben a gazdasejteket károsítanák.

Sikerült ugyan génátvitelt létrehozni rekombináns herpeszvírusvektorok segítségével kísérleti állatok idegrendszerében, de olyan vektorok, melyek nem patogének, hosszú ideig persistálnak a sejtekben és egyenletes szintű génexpresszióra képesek, jelenleg még nem állnak rendelkezésre.[13]

Hepatitiszvírus alapú vektorok kézenfekvő megoldásnak tűnnének rekombináns gének májsejtekbe történő bejuttatására, egyelőre azonban nincs áttörő előrelépés ezen a területen, miután nem megoldott a vírusrészecskék in vitro szaporítása, ugyanakkor a vírus genom különlegesen érzékeny mindenfajta manipulációval szemben.

II. DNS mediálta géntraszfer

A szomatikus génterápia másik elvi lehetősége olyan vektorok alkalmazása, amelyek csupán a bejuttatni kívánt rekombináns gének DNS-ét tartalmazzák. A géntranszfernek ez a fajtája általánosságban az úgynevezett átmeneti (transiens) génátvitel, miután a legtöbb rekombináns molekula gyakorlatilag néhány nap alatt eliminálódik a sejtekből, és a genomba történő stabil integráció csak rendkívül kis számban jön létre. A DNS mediálta géntranszfer alapelve, hogy a létrehozott átmeneti génexpresszió elegendő lehet sok megbetegedés akut fázisának kezelésére, valamint a vírusvektorok használatának kiküszöbölésével olyan, a normálhoz közelálló génstruktúrák alkalmazhatók, melyek jobban tükrözik a génreguláció természetes mechanizmusát.

In vivo DNS mediálta géntranszfert, melynek során DNS-oldatot vagy precipitált DNS-t injectáltak direkt módon a szöve-

1. TÁBLÁZAT

'94 januárjáig az elvégzett *humán* génterápiás *klinikai* kísérletek száma és megoszlása

	Markergén	Terápiás gén
Malignitás	19	30
AIDS	2	4
Májelégtelenség	1	0
ADA-hiány	0	1
Cysticus fibrosis	1	6
Hypercholesterinaemia	0	1
Gaucher-kór	0	3

tekbe sikerrel alkalmaztak izom,[30] máj[32] illetve pajzsmirigy[24] esetében. A kísérletek során a génexpresszió időtartama néhány hónap, illetve néhány nap között váltakozott. Más módszerek esetében a DNS-t lipophil partikulákba „csomagolták",[11] vagy ún. „génpuskát" használtak, ami egy olyan készülék, mely DNS-sel fedett részecskéket lő be a sejtekbe.[33]

A legutóbbi időben sikerült egerek májában génexpressziót létrehozni úgy, hogy a DNS-t egy hordozómolekulához – asyalorosomucoid/polilysin komplexhez – kapcsolták, és ezt a komplexet egyszerű injectio formájában a vénás keringésbe juttatták. Ez a molekula a májsejtek asyaloglycoproteinreceptorához specifikusan kötődik, és a cytoplazmába endocytosis útján kerül be.[26, 31, 32] A létrehozott génexpresszió csupán átmeneti, mivel a DNS illetve produktuma néhány nap alatt eliminálódik a sejtekből. A kísérleti adatok szerint ezen komplex fehérjetermészetű komponensei antigenikusak, kérdésessé téve a többszöri alkalmazás lehetőségét. Megoldást jelenthet olyan, a szervezetben található DNS-t kötő természetes ligandok (pl. laktoferrin) alkalmazása, melyek lehetővé teszik az antitest válaszelkerülését.[15]

Az első, DNS közvetítette génátvitelt alkalmazó klinikai kísérletek során tervezik lipofectin ™-részecskékbe csomagolt, idegenszövet-transzplantációs antigént kódoló DNS in vivo tumorsejtekbe történő befecskendezését, remélve, az idegen gént expresszáló sejtek kilökődését a szervezetből a kiváltott immunválasz révén.

A génterápia területe napjainkban olyan, gyorsan fejlődő része a kutatásnak, mely bizonyos betegségnél reális alternatíva lehet a medicina számára a hagyományos kezelési módok hatástalansága esetén. A módszerek hatékonyságának, biztonságosságának és relatív non-invazivitásának fejlődésével egyre nagyobb lehetőségek tárulnak fel a klinikai alkalmazhatóság számára. Végeredményben, ha sikerül mind az orvosok, mind a betegek számára elfogadható módszereket kidolgozni, a ma még elméleti szinten lévő génterápiás eljárások is realitássá válhatnak a genetikus vagy szerzett megbetegedések széles skálájának kezelésében.

Gene Therapy – Reality or Phantasy?
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SUMMARY

With the advances of molecular genetics clinical trials involving the introduction of recombinant genes into human subjects began in 1989 after a decade of heated debate concerning the technical, social, and ethical implications of somatic gene therapy. This article reviews basic methods for somatic gene therapy and some clinical trials which have been proposed or performed to date. While there will certainly be significant advances in somatic gene therapy in the future, existing methods may be employed fairly in clinical trials.

Key words:
gene therapy, retrovirus, gene transfer, DNA mediated

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