表1　ベーチェット病診断基準（厚生省ベーチェット病調査研究班，1987）

1．主症状
　(1) 口腔粘膜の再発性アフタ性潰瘍
　(2) 皮膚症状
　　a）結節性紅斑
　　b）皮下の血栓性静脈炎
　　c）毛嚢炎様皮疹，痤瘡様皮疹
　(3) 眼症状
　　a）虹彩毛様体炎
　　b）網膜ぶどう膜炎（網脈絡膜炎）
　　c）a），b）を経過したと思われる虹彩後癒着，水晶体上色素沈着，網脈絡膜萎縮，視神経萎縮，併発白内障，続発緑内障，眼球癆
　(4) 外陰部潰瘍
2．副症状
　(1) 変形や強直をともなわない関節炎
　(2) 副睾丸炎
　(3) 回盲部潰瘍で代表される消化器病変
　(4) 血管病変
　(5) 中等度以上の中枢神経病変
3．病型診断の基準
　(1) 完全型：主症状四つ
　(2) 不全型
　　a）主症状三つ（あるいは主症状二つと副症状二つ）
　　b）眼症状＋主症状一つ（あるいは副症状二つ）
　(3) 疑い：主症状の一部が出没
　(4) 特殊病型
　　a）腸管（型）ベーチェット病
　　b）血管（型）ベーチェット病
　　c）神経（型）ベーチェット病
4．参考となる検査所見
　(1) 皮膚の針反応
　(2) 炎症反応
　　赤血球沈降速度の亢進，血清CRPの陽性化，末梢血白血球数の増加
　(3) HLA-B51(B5)の陽性

潰瘍はほぼ必発で，初発症状である場合が多いようです。

皮膚症状としては，下腿に多くみられる痛みをともなう赤い盛り上がった皮疹（結節性紅斑）やにきびの化膿したような皮疹（毛嚢炎様皮疹）がみられます。さらに，皮下の静脈に有痛性の硬結をきたす血栓性静脈炎もよくみられます。また，ベーチェット病では皮膚の被刺激性が亢進しており，虫刺され・外傷などにより容易に化膿する傾向があります。皮膚に針を刺した後に発赤・膿疱を認める“針反応”も同じ機序でおこると考えられ，約50％の患者さんで陽性になります。

眼症状は重要な主症状の一つで，炎症が前眼部のみにおこる虹彩毛様体炎型と，眼底の病変をともなった網膜ぶどう膜炎型に大別されます。前者では，白目の部分が充血し，ときには黒目の部分に白血球が多数たまり前房蓄膿という状態になりますが，視力の低下は軽度です。一方，後者では，自覚的には目の前に霧がかかったようになり，急激な視力低下をきたします。

外陰部潰瘍は，陰茎，陰嚢，小陰唇，膣壁などにできる口腔内アフタに似た境界鮮明の有痛性の潰瘍で，ひどい場合には，鼠径部の皮膚にも潰瘍を生じることがあります。外陰

部潰瘍は，一般に発病初期に多くみられますが，口腔内アフタに比べて再発は少ないようです。

そのほか副症状としては，関節炎，副睾丸炎，消化器病変（腸管ベーチェット），血管病変（血管ベーチェット），神経病変（神経ベーチェット）が認められますが，いずれの症状も頻度は主症状ほど多くはありません。

どう治療するか

1）治療の基本的な考え方

重篤な視力障害を残しうる眼病変，生命予後に影響を及ぼす特殊病型（神経・血管・腸管ベーチェット）に対しては積極的な薬物療法を行なう必要がありますが，口腔内アフタ，外陰部潰瘍，皮膚病変に対しては副腎皮質ステロイドの外用を中心とした局所療法での対応でも十分です。しかし，疼痛の強い場合や発作の頻度が多い場合には全身的薬剤投与を行ないます。この場合はコルヒチン，非ステロイド抗炎症薬（NSAID）が中心となります。

ベーチェット病では白血球（好中球）の機能が亢進していることが知られており，コルヒチンはこの好中球機能を抑制することから，ベーチェット病の治療薬として頻用されますが，副作用として下痢，乏精子症，月経異常，催奇性，筋症状（こむらがえり）に注意する必要があります。NSAIDの運用にあたっては，副作用としてとくに消化性潰瘍に対する注意が必要です。また，皮膚・粘膜病変に対しては，イコサペント酸エチルが有効な場合があり，副作用も少なく，試みる価値はあります。

副腎皮質ステロイドの全身投与は，ベーチェット病の急性炎症を短期的に軽快させる効果がありますが，持続的に長期使用してもベーチェット病の各症状の新たな発作を抑制する効果はありません[3]。また，逆に眼病変のある場合に急に副腎皮質ステロイドを減らすと新しい眼発作が誘発されるので，注意が必要です。

2）日常生活の管理

ベーチェット病においては，気象条件，感染，手術，外傷，月経，ストレスが増悪因子となることが多いので，避けることのできるものは避けるようにすることが大事です。とくに，う歯やそのほかの感染巣（皮膚の膿瘍など）がある場合はかならずその治療を行なっておく必要があります。さらに，毎食後かならず歯磨きと口腔内の洗浄を欠かさないようにすることが，口腔内アフタの予防にもつながります。喫煙は慢性進行型の神経ベーチェットの危険因子であり，そのほかの病変にも悪影響を及ぼすと考えられるので，中止することが大切です[4]。

3）治療の実際

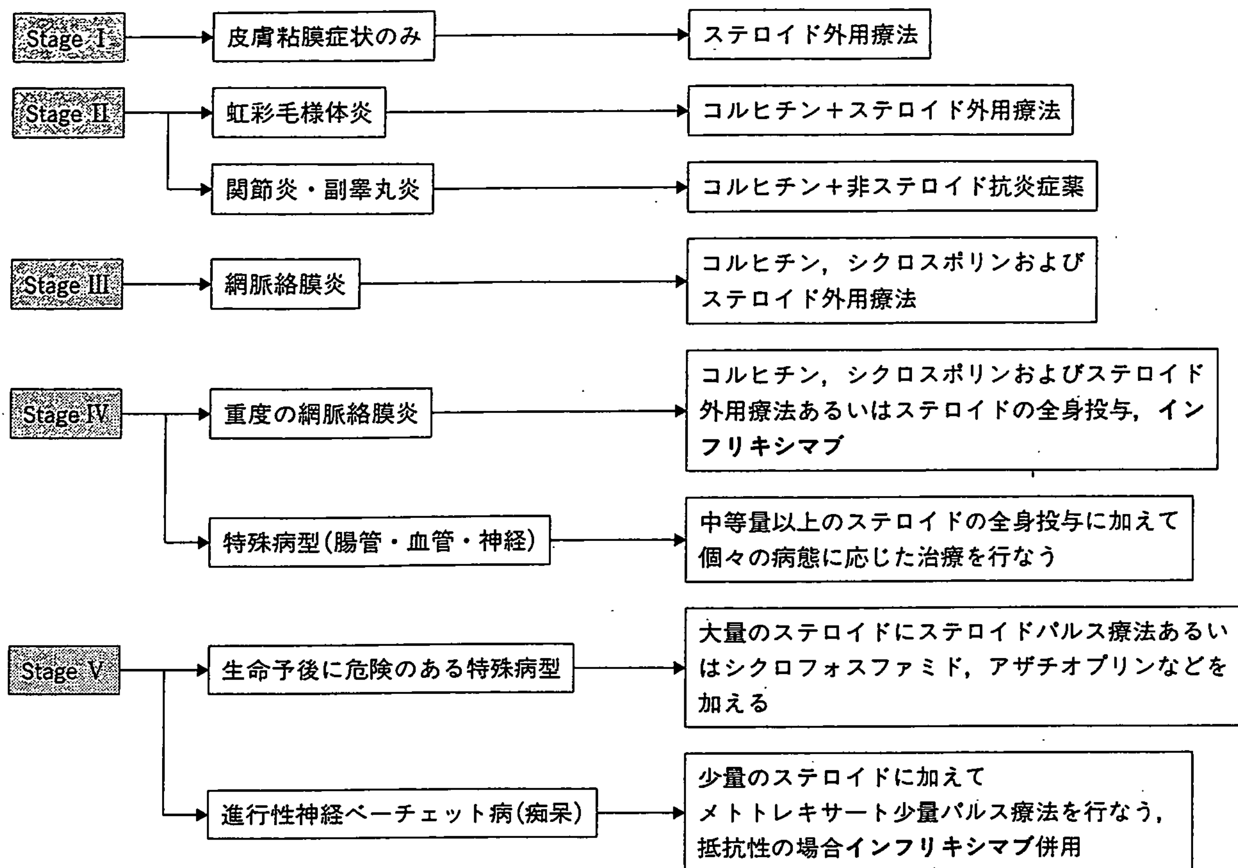
2003年にベーチェット病の重症度分類基準が厚生労働省の研究班により示され，これによって大まかな治療方針を決定することができます（図1）。

①眼病変

発作が生じた場合にはステロイド薬の点眼・局所（結膜下）注射を行ないます。しかし，眼病変を繰り返すことにより，視力低下ひいては失明の危険がありますので，発作を繰り返さないようにすることが大切です。

発作の予防を目的としては，まずコルヒチンの全身投与が一般的に行なわれます。この際，コルヒチンで効果が不十分な場合は，シクロスポリン（ネオーラル）と呼ばれる免疫抑制剤の治療が行なわれます。シクロスポリンは，副作用を予防するという目的で，血中濃度（トラフ値と呼ばれる服薬直前の最低値）を100～200 ng/mℓに保つように投与量が調節されます。シクロスポリンの副作用としては腎障害がもっとも多くみられますが，

図1 ベーチェット病の重症度分類とそれに応じた治療方針



Stage ⅠからⅤにいくにしたがって重症となる

ベーチェット病の患者さんに特有な副作用として，神経障害（髄膜脳炎様症状）に注意する必要があります。シクロスポリン内服中に，頭痛・発熱をきたした場合には，まずこの副作用を疑ってみる必要があります。

難治性の眼病変に対しては，抗TNF-α抗体（インフリキシマブ）が有用であることが証明され[5]，2007年1月に保険適応になっています。

②神経・血管・腸管病変

これらの特殊病型と呼ばれるものは，生命にかかわることが多く，積極的な治療が必要です。基本治療薬であるコルヒチンに加えて，中等量〜大量の副腎皮質ステロイドの全身投与（プレドニゾロン 30〜60mg/日相当）が行なわれます。症状が軽快し安定したら副腎皮質ステロイドを減量していきますが，急激な減量により，原病のみならず眼病変の増悪を誘発することもあるので，注意が必要です。一部の患者さんにみられる慢性進行型の痴呆・小脳症状を主体とした難治性の神経ベーチェットに対しては，メトトレキサートの少量パルス療法が有効であることが示されています[2]。

血管病変に対してはワーファリン，アスピリン（小児用バファリン）など血液凝固を抑制する薬剤の投与を併用することもあります。腸管病変に対しては，副腎皮質ステロイドに加えてサラゾスルファピリジン（サラゾピリン）やメサラジン（ペンタサ）の投与が併用されることがあります。また，血管病変や腸管病変においては外科的治療が必要となる場合もあります。

今後の展望

ベーチェット病の眼病変に対する治療は，インフリキシマブの登場で画期的に改善されていくものと期待されます。しかし，特殊病型，なかでも慢性進行型の神経ベーチェットは今なお難治性であり，患者さんを廃人同様にしてしまう点で憂慮すべき病態です。こうした難治性病態に対してもインフリキシマブの効果が期待されています。

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［ひろはた・しゅんせい／膠原病・感染内科］

特集 第20巻第11号
整形外科医のためのcareとcure-II
—RAのtight controlを目指して—

【整形外科医のための標準薬物治療の基礎知識】

ブシラミン—まだまだ存在価値があるtraditional DMARD—

Bucillamine; A valuable traditional DMARD

廣畑 俊成*
Shunsei Hirohata

はじめに

ブシラミン(bucillamine; BUC)は本邦で開発されたSH化合物であり，メトトレキサート(methotrexate; MTX)とともに関節リウマチ(rheumatoid arthritis; RA)に対する有効な疾患修飾性抗リウマチ薬(disease modifying antirheumatic drug; DMARD)としての地位を確立したといっても過言ではない．本稿においては，BUCの薬理作用，有効性，副作用，使い方などについて概説するとともに，MTXとBUCの併用効果についても触れてみたい．

I．薬理作用

1．BUCの構造上の特徴

D-ペニシラミン(D-penicillamine; DPC)とBUCはいずれも分子内にSH基を有するいわゆるSH化合物である(図1)[1]．事実，この両薬剤の効果発現までの期間・臨床効果・副作用などについては類似している点も多い．しかし，この両薬剤は構造上決定的な違いを有している．すなわち，DPCは分子内にSH基を1つしかもたないのに対し，BUCは同一分子内にSH基を2つ有しているという点である．このために，BUCを投与された患者においては生体内でいくつかのユニークな代謝産物が形成さることが明らかになっている．その中には，2つのSH基が分子内S-S結合を形成したSA981，2つのSH基のうちの1つがメチル化されたSA679，SH基が2つともメチル化されたSA672が含まれる[1]．このような代謝経路以外にも，DPC・BUCは分子間でS-S結合を形成することが知られている．

2．BUCの薬理作用

1) リンパ球に対する作用

関節リウマチにおいては，リウマトイド因子(以下RF)や抗CCP抗体などの自己抗体が出現し，その病態形成にあたりBリンパ球が重要な役割を果たすことが推察される．

図2に*in vitro*におけるBUCの各種代謝体とDPC(すべて50 μg/ml)のヒトBリンパ球に対する作用を示した[1]．BUCは銅イオン

Key words▶
SH化合物 (Sulfhydryl compounds)
D-ペニシラミン (D-penicillamine)
メトトレキサート (methotrexate)
併用療法 (combination therapy)

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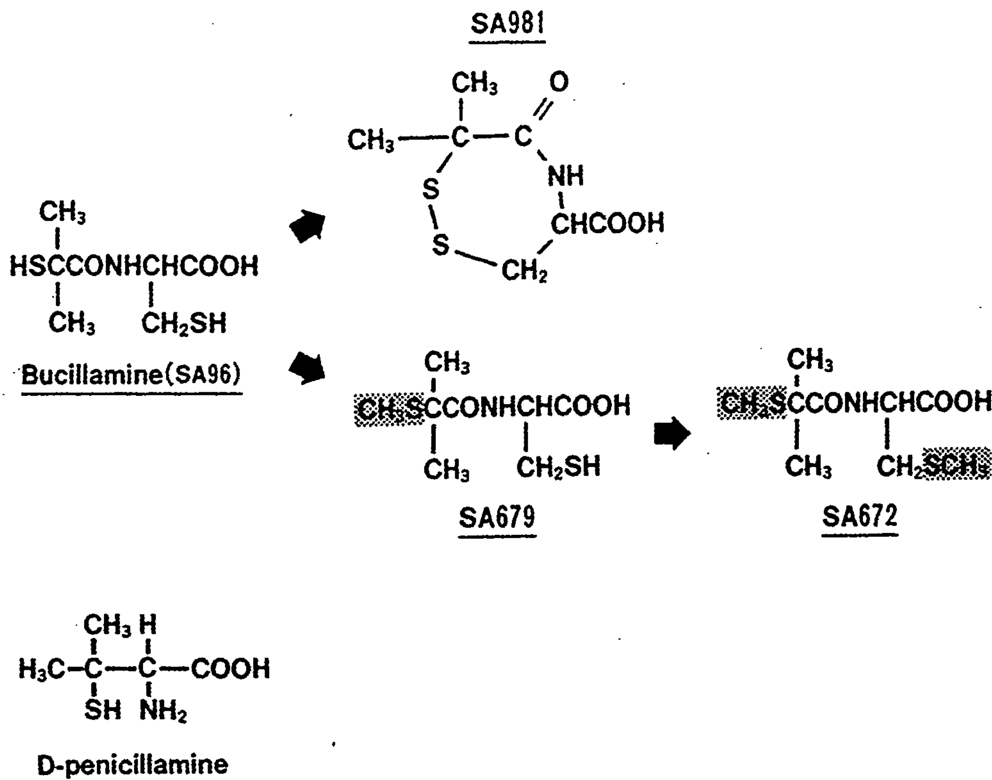

図1　DPC，BUCおよびその代謝体の化学構造式

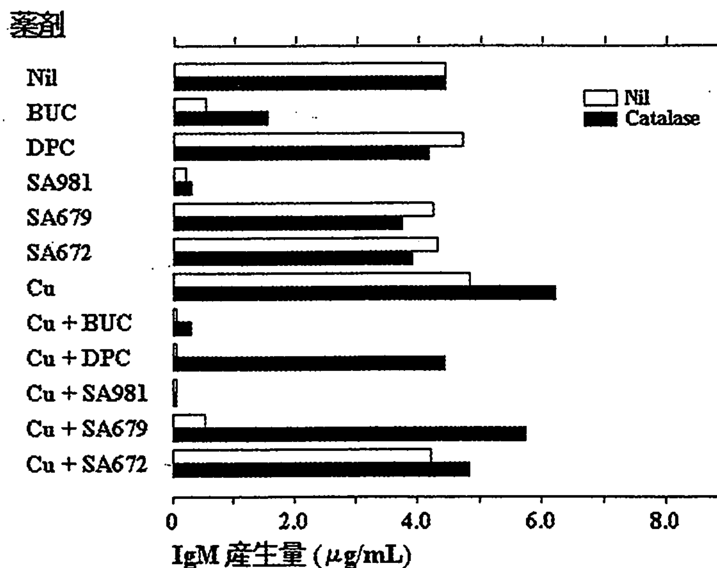

図2　DPC，BUCおよびその代謝体のBリンパ球によるIgM産生の抑制効果の比較
SA+IL-2によりBリンパ球を刺激し，IgM産生を誘導，薬剤はすべて50 μg/mlで添加．$CuSO_4$ (5 μg/ml)，カタラーゼ(10 μg/ml)を適宜添加．（文献1より引用）

の非存在下においても*Staphylococcus aureus* (SA)＋インターロイキン(IL)-2により刺激されたBリンパ球のIgM産生を抑制したが，銅イオンの添加によりその抑制作用はさらに増強した．カタラーゼはBUCのIgM産生抑制作用を部分的に解除した．これに対して，DPCは銅イオンの存在下においてのみBリンパ球のIgM産生を抑制し，この抑制効果

はカタラーゼの添加により完全に消失した．SA679はDPCと全く同様の抑制効果を示したが，SA672には全くBリンパ球の抑制効果が認められなかった．注目すべき点は，分子内S-S結合を有するSA981は銅イオンの存在・非存在にかかわらず強いIgM産生抑制作用を示し，しかもこの抑制効果がカタラーゼにより全く影響を受けなかった．

BUCとDPCの免疫調節作用についての重要なポイントは以上に示したデータに集約されている．すなわち，①BUCにおいてもDPCにおいてもフリーのSH基の存在がその作用上必須であり，これは主として銅イオンとの相互作用により生じた過酸化水素などの活性物質を介して働く，②BUCの代謝体であるSA679の作用はDPCの作用とほぼ同一である，③分子内S-S結合を有するSA981は独特の強力なBリンパ球の抑制作用を有し，この代謝体の形成がBUCのユニークな作用を支えている．さらに，*in vivo*でもBUCからSA981への転換が起こっていることは確認されており，これがBUCの銅イオン非依存性の抑制作用を支えているものと考えられる．

2）骨髄細胞に対する作用

近年，RAの病態形成において骨髄の異常が関与することが報告されている[2]．RA関節滑膜には，マクロファージ様のA型滑膜細胞と線維芽細胞様のB型滑膜細胞が存在し，このいずれもが骨髄CD34$^+$細胞より分化しうることがわかっている[2]．MTXは骨髄CD34$^+$細胞からのA型滑膜細胞様細胞の分化を阻害すると考えられるが，BUCにはこうした作用は認められない[2]．逆に，SA-981は骨髄CD34$^+$細胞からのB型滑膜細胞様細胞の分化を阻害すると考えられるが，MTXにはそうした抑制作用はないようである[2]．したがって，これらの*in vitro*のデータよりMTXとBUCの併用による相乗的治療効果が十分期待されるところである．

Ⅱ．有効性

BUCのRAに対する有効性は臨床治験により実証されているが，プラセボとの二重盲検試験では投与8週間目より有意差がみられている[3]．一方で，自験例の中には投与後6カ月より効果が著明に出現し始めたものもあることから，副作用のない限り少なくとも6カ月間は投薬を継続するべきであろう．

RA患者を対象とした5年間にわたる長期投与試験ではBUCは20.6％の患者で5年間にわたり有効性を示し，投与継続率は他のDMARDとほぼ同等であることが報告されている[4]．しかし，この報告でのBUCの初期投与量が300 mg/日であり，かつ副作用による中止例が23.5％であったことから，初期投与量を100mg/日としていた場合は5年間の継続率が上昇していた可能性が示唆され[4]．事実，三橋らの報告ではBUCの5年間の投与継続率は37.1％であり，すべての例は200 mg/日以下であったという[5]．したがって，副作用による投与中止を避けるためにも，BUCは50～100 mg/日より始めて，最大200 mg/日まで増量するという方法が推奨されている．

BUCは注射金剤・経口金剤の無効例や効果減弱例に対しても有効であることが数多く報告されている．また，DPC無効例に対してもBUCが有効であることが多いが，これは前述したように同じSH基剤であるDPCともBUCの作用機序は異なることより理解できる[6]．

表1　ブシラミン(BUC)による顆粒球減少

1. 投与開始後1カ月前後での発症が多い
2. 感冒様症状(発熱・咽頭痛)が前駆症状としてみられることが多い
3. 進行が急激である
4. 投与中止で徐々に回復する
5. G-CSF使用時は顆粒球が増え過ぎないように十分注意する

表2　ブシラミン(BUC)による腎障害

1. 投与開始後6カ月以内での発症が多い
2. 用量依存性の傾向がある(DPCでは用量依存性が証明されている)
3. 膜性腎症の組織像をとることが多い
4. 休薬で陰性化しない場合，プレドニゾロン30 mg/日程度の投与により完全に陰性化する

表3　ブシラミン(BUC)による肺障害

1. 高齢者に多い
2. リウマトイド因子(RF)高値陽性例が多い
3. 投与開始後3カ月前後での発症例が多い
4. RAに対するブシラミン有効例に多い
5. 初発症状では乾性咳嗽がみられる
6. 胸部X線上ではmottled型(まだらな浸潤影，索状影)を示すことが多い
7. 投与中止のみで軽快することもあり，必ずしも直ちにステロイドの投与が必要なわけではない

Ⅲ. 副作用

BUCはDPC同様SH基をもっていることから，DPC類似の副作用がみられる．主な副作用として，過敏症(皮膚症状)，ネフローゼ症候群(蛋白尿)，肝障害，白血球減少(特に顆粒球減少)，間質性肺炎ならびに胃腸障害などがある．

1. 白血球減少(顆粒球減少)（表1）

BUCによる顆粒球減少はDPCと同様，投与開始後1カ月前後で発症する例が多い．また発症した場合進行が急激であるため，この期間に必ず血液検査を行い顆粒球の変動を確認することが大切である．特に，顆粒球減少の前駆症状としてみられる発熱や咽頭痛の発現には注意を要する．白血球の減少は，本剤の投与中止により徐々に回復するが，1,000/mm^{3}台に低下したり感染症がある場合にはG-CSF(granulocyte colony-stimulating factor)を使用することになる．血小板減少をきたすこともある．

2. 腎障害(ネフローゼ症候群)

BUCによる腎障害の特徴を表2に示す．蛋白尿はBUCの用量が多くなるほど出現しやすい．したがって後述するように，BUCの投与量は200 mg/日までにとどめておく方が無難であると考えられる．DPCによる腎障害と同様に，組織学的には膜性腎症の病理像を示す．BUCの投与を中止するだけでは改善しないことが多い．このような場合には，

ステロイドの投与もしくは増量(プレドニゾロン30 mg/日程度)によりすみやかに改善することが多い.

3. 肺障害(表3)

BUCによる肺障害は，高齢者あるいはRF高値の症例に発現することが多く，投与後3カ月前後の発症例が多いとされる[7]. また，RAに対する有効例に多い[7]. 初発症状では乾性咳嗽がみられる. 胸部X線上ではまだらな浸潤影・索状影を示すことが多い. 投与中止だけで肺炎が消失することもあり，必ずしも直ちにステロイドの投与が必要なわけではない. 発症様式は潜行性で徐々に進行していき，投与を中止すると改善がみられる. 致命的な症例は報告されておらず，その意味ではMTX肺炎と対照的である.

Ⅳ. 使い方

BUCが一般臨床に使用され始めた当時，BUCによる治療は添付文書どおり300 mg/日(100 mg錠を1日3回，毎食後に経口投与)で開始されていた. しかし，300 mg/日では副作用の発現頻度が上昇することが明らかになり[8]，現在では1日100 mgより投与を開始し，増量していく方法が推奨されている. しかし，1日300 mgまで増量されることは少ない. これは，BUCの効果と副作用の発現とのバランスに基づいている. 逆に，最近では1日50mgでも良好な効果が得られることが明らかになってきており，50 mg錠も用いられている.

現在，BUCの1日平均処方量は約200 mgである. 投与中に副作用の発現がみられた場合には，減量または休薬して経過観察する.

動物実験でBUCによる催奇性は認められていないが，妊婦の安全性に対する報告はないため，投与は避ける. なお，血液障害，骨髄機能低下，腎障害のある患者に対しては投与禁忌である.

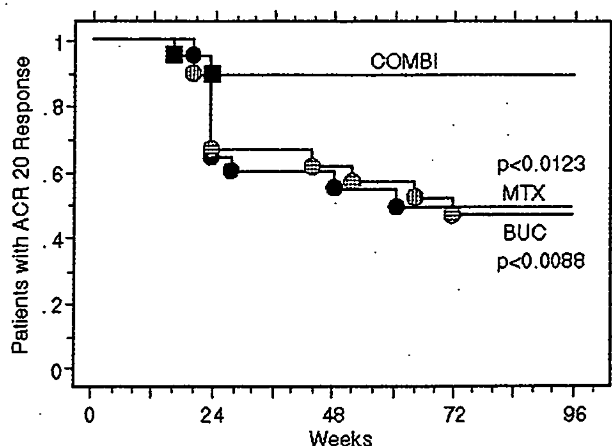

図3 BUCとMTXの併用効果
BUCとMTXの併用群ではおのおのの単独群よりもACR20の維持率が有意に高い(文献9より引用)

Ⅴ. MTXとの併用療法

市川らによりRAに対するBUCとMTXの併用効果が検討された結果，BUC+MTXの併用はおのおのの単剤投与に比較して，24カ月後のACR20の改善率が79%と有意に高かったことが報告されている[9]. さらに，いったん達成したACR20の継続率もBUC+MTX併用群で有意に高いことが示された(図3)[9]. 前述したように，BUCとMTXはRA骨髄CD34⁺細胞に対する作用が異なり，両者の併用によりA型滑膜細胞とB型滑膜細胞の両者の分化を阻害し得る可能性が示唆されている. このように，BUC+MTXの併用効果については*in vitro*のデータからも裏づけられている[2]. しかし，実際的に使用する場合には，BUCとMTXの両者を同時に開始すると，副作用の出現した際にいずれの薬剤に起因したものかの同定に苦慮する可能性がある. したがって，まず片方の薬剤より開始して，副作用のないことを確認したうえで併用を行う方

が無難であろう．

おわりに

以上，本邦で開発されたBUCの作用機序・効果・副作用・使用法を中心に概説した．特に，MTXとの併用療法のおのおのの単剤による治療に対する優位性が証明されたことより，生物学的製剤に次ぐ有力な治療法であると期待される．BUCの至適用量は100～200 mg/日であるが，本邦でのMTXの保険上の使用量の上限は8 mg/週と欧米の50％であり，至適とはいえない．今後，MTXのより高用量との併用についても検討していく必要があると考えられる．

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Characterization of an exchangeable gene trap using pU-17 carrying a stop codon-*βgeo* cassette

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We have developed a new exchangeable gene trap vector, pU-17, carrying the intron-*lox*71-splicing acceptor (SA)-*βgeo*-*lox*P-pA-*lox*2272-pSP73-*lox*511. The SA contains three stop codons in-frame with the ATG of *βgalactosidase/neomycin-resistance fusion* gene (*βgeo*) that can function in promoter trapping. We found that the trap vector was highly selective for integrations in the introns adjacent to the exon containing the start codon. Furthermore, by using the Cre-mutant *lox* system, we successfully replaced the *βgeo* gene with the *enhanced green fluorescent protein* (*EGFP*) gene, established mouse lines with the replaced clones, removed the selection marker gene by mating with Flp-deleter mice, and confirmed that the replaced *EGFP* gene was expressed in the same pattern as the *βgeo* gene. Thus, using this pU-17 trap vector, we can initially carry out random mutagenesis, and then convert it to a gain-of-function mutation by replacing the *βgeo* gene with any gene of interest to be expressed under the control of the trapped promoter through Cre-mediated recombination.

Key words: Cre/*lox*, embryonic stem cell, Flp/*FRT*, gene trap, site-specific recombination.

Introduction

The whole human and mouse genome sequences are now near to completion (Waterston *et al.* 2002). However, gene functions *in vivo* cannot be understood from the sequence information alone, and mutational analysis is a powerful and efficient approach for studying functional genomics. Gene trapping in embryonic stem (ES) cells is a proven method for isolating large numbers of random insertional mutations that can be easily identified (Gossler *et al.* 1989; Gossler 1993; Evans *et al.* 1997; Zambrowicz & Friedrich 1998; Stanford *et al.* 2001; Hansen *et al.* 2003; Stryke *et al.* 2003). Gene trap vectors contain a promoter-less reporter gene downstream of a splice acceptor (SA) and a selectable marker gene. When the gene trap vector is introduced and integrated into endogenous genes, a fusion transcript between the endogenous gene and the reporter gene is produced, such that the expression of the trapped gene can be monitored. Both the trapped cDNA and the genomic site of the integration can easily be cloned by rapid amplification of cDNA 5′-ends (5′-RACE) (Townley *et al.* 1997) and the plasmid rescue method (Araki *et al.* 1999). To date, trap vectors carrying the internal ribosomal entry site (IRES) from the encephalomyocarditis virus (ECMV) (Jang & Wimmer 1990; Ghattas *et al.* 1991; Mountford & Smith 1995; Kang *et al.* 1997) and the *βgalactosidase/neomycin-resistance fusion* gene (*βgeo*) (Friedrich & Soriano 1991; Voss *et al.* 1998) have been widely used and proven to trap various genes expressed in ES cells (Chowdhury *et al.* 1997; Bonaldo *et al.* 1998; Stoykova *et al.* 1998).

In typical gene trapping, insertion of a trap vector can only induce truncation mutations. In order to change the trapped alleles into a more subtle mutation, such as a point mutation, we previously developed a site-directed integration system in ES cells using the Cre-LE/RE mutant *lox* system (Araki *et al.* 1997), and constructed an exchangeable gene trap vector, pU-Hachi, carrying SA-*lox*71-IRES-*βgeo*–polyadenylation signal (pA)-*lox*P-pA-pUC (Araki *et al.* 1999). The *βgeo* gene in pU-Hachi trap clones can be replaced with any other cDNA of interest through Cre-mediated integration.

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Therefore, we can carry out random insertional mutagenesis as the first step, and then introduce cDNA fragments for expression in the same pattern as the *βgeo* in the second step. Thus, we can also utilize trap clones as promoter resources.

However, there are several limitations to replacement experiments using pU-Hachi. First, the SA and *lox*71 sequences contain 4 and 1 stop codon(s), respectively. Hence, if a genomic gene carrying both exons and introns is inserted into the *lox*71 site, the stop codons present in the SA become premature translation termination codons, leading to nonsense codon-mediated mRNA decay (NMD) (Wagner & Lykke-Andersen 2002). Second, because the IRES is used for cap-independent translation of the *βgeo* gene, it is necessary to use the IRES for translation of the inserted cDNA and this makes the construction of replacement vectors laborious. Third, trap vectors containing the IRES often integrate into the 3′ region of a gene resulting in the production of a truncated form, which could modify the phenotype (Oike *et al.* 1999). Fourth, the 3′-part of the trapped gene cannot be reutilized, because the pA sequence is located outside the floxed region and cannot be removed. For example, we have tried 3′-rapid amplification of cDNA ends by inserting a promoter sequence, but no transcripts fused to the 3′-part of the trapped gene were produced. Fifth, it is difficult to insert and express the *cre* gene, because a re-excision reaction sometimes occurs in the LE/RE mutant *lox* system (Albert *et al.* 1995).

Recently, we showed that combination of the LE/RE mutant *lox* with a heterospecific *lox*, *lox*2272, improved the recombination efficiency and made it possible to integrate the *cre* gene through recombination by Cre (Araki *et al.* 2002). Based on these findings, we have constructed a new gene trap vector suitable for the insertion and expression of cDNA, genomic DNA and the *cre* gene. The new gene trap vector, pU-17, was designed to be a promoter trap and carried 3 kinds of mutant *lox* sites for replacement. We demonstrate here that pU-17 is efficiently integrated around the initiation codon, the *βgeo* gene is easily replaced with the *enhanced green fluorescent protein* (*EGFP*) gene, and the same expression pattern *in vivo* is maintained after the replacement.

Materials and Methods

Plasmids

The trap vectors pU-17 and pU-18 were constructed from the pU-Hachi vector by several modifications (Araki *et al.* 1999):

1. The *lox*71 site was inserted to the 5′-side, within the intron sequence of the mouse *En-2* gene.
2. The polyadenylation (pA) signal of the *β*geo gene was removed.
3. A *lox*2272 sequence was inserted in front of the pA signal of the mouse *phosphoglycerate kinase-1* (*Pgk*) gene.
4. The pSP73 (Promega, Madison, WI, USA) vector was used instead of the pUC vector in pU-Hachi.
5. The IRES sequence was removed. The vectors were linearized at their single *Spe*I site before electroporation.

The Cre expression vector, pCAGGS-Cre, was described previously (Araki *et al.* 1995; Araki *et al.* 1997). The plasmid pCAGGS-Flp was constructed by ligating fragments of the *Flp* gene (Stratagene, La Jolla, CA, USA) into the *Eco*RI sites of pCAGGS. The mutation in the *Flp* gene (Ringrose *et al.* 1998) was corrected.

The replacement vector, p6SEFPPF, was assembled from components of pSP73 (Promega), the *lox* 66 sequence, the *EGFP* gene (Clontech, Palo Alto, CA, USA), the *FRT* sequence, the *Pgk* promoter, the *puromycin N-acetyltransferase* (*Pac*) gene and the *lox*P sequence.

Cell culture and electroporation

The ES cell lines TT2 (Yagi *et al.* 1993) and E14tg2a (Niwa *et al.* 2002) were grown as described. For electroporation with the pU-17 and pU-18 gene trap vectors, 80 μg of *Spe*I-digested DNA and 2×10^7 cells were used. The cells were suspended in 0.8 mL of phosphate-buffered saline (PBS), electroporated using a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Hercules, CA, USA) set at 800 V and 3 μF, and then fed with medium supplemented with 200 μg/mL of G418 after 48 h. Selection was maintained for 7 days, and the colonies were then counted, picked and placed in 24-well plates. Replacement by Cre-mediated recombination in ES cells was performed as described previously (Araki *et al.* 1999).

Analyses of genomic DNA

Cells or tissues were lysed with sodium dodecylsulfate (SDS)/proteinase K, treated with 1:1 (v/v) phenol/chloroform, precipitated with ethanol, and dissolved in 10 mM Tris-HCl, pH 7.5/1 mM ethylenediamine tetraacetic acid (EDTA) (TE). Six micrograms of genomic DNA was digested with appropriate restriction enzymes, electrophoresed in a 0.9% agarose gel and then blotted onto a nylon membrane

(Roche Diagnostics, Basel, Switzerland). Hybridization was performed using a DIG DNA Labeling Kit (Roche).

For polymerase chain reaction (PCR) analysis, DNA (50 ng) were subjected to 30 cycles of amplification (with each cycle consisting of 1 min at 94°C, 2 min at 55°C and 2 min at 72°C) in a thermal cycler. The primer sequences were as follows: for detection of the recombined allele, SA5 (5′-GGTCACTTTATGTTCTTGCCC-3′) and GFP2 (5′-TGTGATCGCCGTTCTCGTTG-3′); for detection of the *βgeo* sequence, Z1 (5′-GCGTTACCCAACTTAATCG-3′) and Z2 (5′-TGTGAGCGAGTAACAACC-3′); for detection of the *CAGGS-Flp* transgene, AG2 (5′-CTGCTAACCATGTTCATGCC-3′) and Flp5 (5′-ATCCTACCCCTTGCGCTAAA-3′).

RNA analyses

Total RNA was isolated from ES cells using Sepasol (Nakalai, Kyoto, Japan). Ten micrograms of total RNA was electrophoresed through 1.0% agarose-formaldehyde gels and transferred to a positively charged nylon membrane (Roche). After baking at 80°C for 1 h, the membrane was prehybridized, and then hybridized with RNA probes prepared using a DIG RNA Labeling and Detection Kit (Roche).

Five micrograms of total RNA was used for first-strand cDNA synthesis with the reverse transcriptase ReverScript (Wako Pure Chemical Industries, Osaka, Japan) and the LZUS3 primer (5′-GCGCATCGTAACCGTGCAT-3′) in the lacZ sequence. 5′-RACE was performed using the 5′-RACE system (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The initial PCR was performed using the primer SA13 (5′-TCTGAAACTCAGCCTTGAGC-3′) in the SA sequence and the anchor primer (5′-GGCCACGCGTCGACTAGTACGGGiiGGGiiGGGiiG-3′) (Invitrogen). Next, nested PCR was performed using the primer SA10 (5′-AGCAGTGAAGGCTGTGCGA-3′) in the SA sequence and the amplification primer (5′-GGCCACGCGTCGACTAGTAC-3′) in the anchor primer sequence. The PCR product was electrophoresed, purified with Quantum Prep Freeze 'N Squeeze DNA Gel Extraction Spin Columns (Bio-Rad), and sequenced by the dideoxy-chain termination method using a Big Dye Terminator Cycle Sequencing kit (Perkin Elmer, Foster City, CA, USA). All the obtained sequences were confirmed by RT-PCR using first-strand cDNA synthesized with random primers. The obtained sequences were compared with the GenBank and GenEMBL databases using the BLASTN program (http://blast.genome.jp) (Altschul *et al.* 1990), and the exon-intron structures were examined using the Celera Discovery System (Applied Biosystems Japan, Tokyo, Japan).

Production of chimeric mice and microinjection

Chimeric mice were produced by aggregation of ES cells with eight-cell embryos of ICR mice (CLEA Japan, Tokyo, Japan). Chimeric male mice were mated with C57BL/6 J females (CLEA Japan) to obtain F1 heterozygotes.

For microinjection of CAGGS-Flp, superovulated BDF1 (Charles River, Osaka, Japan) females were mated with BDF1 males. Fertilized eggs were collected and pronuclear injection was performed according to a previously described procedure (Yamamura *et al.* 1984).

Histological analysis

For 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) staining, tissues were fixed in 4% paraformaldehyde for 6 h, sectioned with a vibratome at 50 μm, treated with 1% Triton X-100 in PBS for 10 min, washed three times in PBS, incubated overnight at 30°C in staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.5% X-gal in PBS), mounted on glass slides, and counterstained with Nuclear Fast red. For immunohistochemistry, paraffin sections were prepared and stained with an anti-EGFP rabbit polyclonal antibody (MBL, Nagoya, Japan).

Results

Construction of pU-17

The structure of pU-17 is shown in Figure 1(A). The improvements from pU-Hachi are as follows:

1. A *lox*71 site was inserted into the intron sequence of the SA.
2. *Lox*2272 and *lox*511 sites were inserted into the downstream of the pA and plasmid vector tail, respectively. Through these two alterations, various replacements became possible (see Fig. 5 and Discussion).
3. The IRES was removed, and as a result, the three stop codons in the exon sequence of the SA were in-frame with the ATG of the *βgeo* gene (Fig. 1C). It is theoretically expected that only clones in which the vector is integrated into the upstream of the start codon of a trapped gene will become neo-resistant. Thus, this Stop-*βgeo* vector is expected to function in promoter trapping, and should be ideal for expressing cDNA under the control of a trapped gene. However, this restriction of the integration site may result in a reduction in the colony formation efficiency. Therefore, we examined the colony formation efficiency of pU-17 by comparison with a trap vector using the IRES of ECMV.

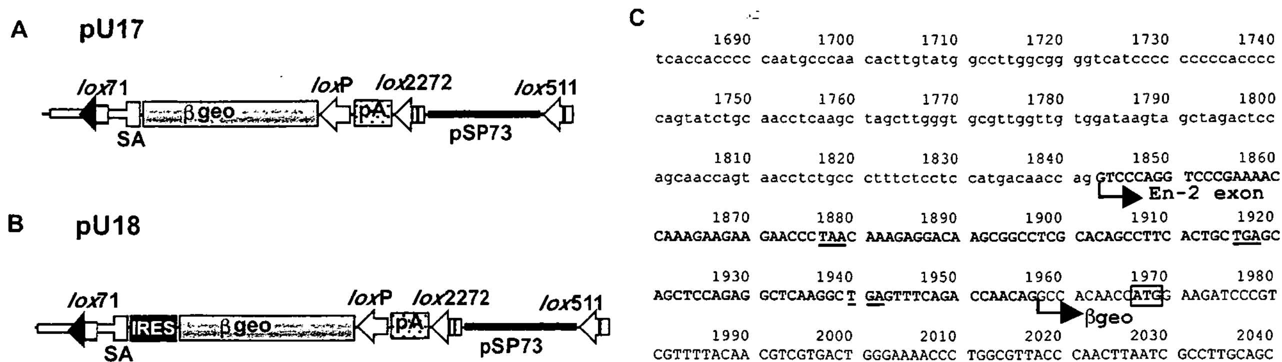

Fig. 1. Structure of the gene trap vectors. (a) Schematic representation of the trap vector pU-17. pU-17 contains 1.8 kb of an intron and a splice acceptor (SA) sequence from the mouse *En-2* gene, the *βgeo* gene and a polyadenylation signal (pA). A *lox*71 site is located within the intron sequence, and *loxP*, *lox*2272 and *lox*511 sites are placed in the downstream of the *βgeo*, pA and pSP73 vector sequences, respectively. (b) Schematic representation of the trap vector pU-18. The only difference between pU-17 and pU-18 is the presence of the internal ribosomal entry site (IRES) sequence of encephalomyocarditis virus (ECMV) between the SA and *βgeo*. (c) Sequence of the junction of the SA and the *βgeo* gene of pU-17. Lower and upper case letters represent the intron and exon sequences, respectively. There are three in-frame stop codons (underlined) in the upstream of the *βgeo* initiation codon (boxed). It is theoretically expected that the integration site of the vector should be restricted to the upstream of the start codon of a trapped gene.

Table 1. Colony formation efficiencies of pU-17 and pU-18

	Number of G418-resistant colonies		
Trap vector	1st exp.	2nd exp.	3rd exp.
pU-17	34	135	36
pU-18	38	153	42

Eighty micrograms of linearized trap vector was introduced into 2×10^7 E14Tg2a cells. The electroporated cells were plated onto three 10 cm dishes and selected with G418 for 1 week, before the number of colonies was counted.

pU-17 has comparable colony formation efficiency to a trap vector containing an IRES

We constructed the pU-18 vector (Fig. 1B) carrying the IRES between the SA and *βgeo*. The only difference between pU-17 and pU-18 is the absence and presence of the IRES, respectively. In pU-18, a fusion message of the trapped gene and IRES-*βgeo* is produced, and translation starts from the AUG of both the trapped gene and *βgeo*. Therefore, trap clones should be G418-resistant independent of the insertion sites of the IRES-*βgeo* vector within the trapped genes (Bonaldo *et al.* 1998). The two vectors were introduced into ES cells harvested on the same day through electroporation. The cells were then selected with G418, and the numbers of colonies were counted. As shown in Table 1, almost the same numbers of colonies appeared with both vectors, indicating that the pU-17 Stop-*βgeo* vector has comparable colony formation efficiency to pU-18.

pU-17 integrates into the 5′-region of trapped genes

In order to examine the integration sites of pU-17 in trap clones, we first performed Northern blotting using randomly chosen pU-17 and pU-18 trap clones to compare the sizes of the fusion messages. If pU-17 integrates in the upstream of the start codon of a trapped gene, the lengths of trapped mRNA fused to *βgeo* should be short and therefore the total lengths of the fusion messages in pU-17 clones should be about 4.5 kb, corresponding to the length of SA-*βgeo*-pA. As shown in Figure 2(A), the sizes of the fusion messages in pU-17 clones were similar, spanning approximately 4.5–5 kb. On the other hand, various sizes of bands, most of which exceeded 5 kb, were detected in pU-18 clones, suggesting integration in the middle or 3′-half of the genes. This result indicates that pU-17 tends to integrate into the 5′-region of endogenous genes.

Next, to precisely analyze the insertion events in a larger number of trapped clones, trapped genes containing the pU-17 vector were determined by 5′-RACE analysis, and the results are summarized in Table 2. Regarding the 22 clones in which pU-17 was integrated into known genes, the integration sites in the context of their exon-intron structures were determined using the Celera Discovery System. As shown Figure 2(B), in 82% of the trap clones, pU-17 was integrated into the introns adjacent to the exon containing the start codon of the trapped gene. This results indicates that the pU-17 functions efficiently as a promoter trap vector.

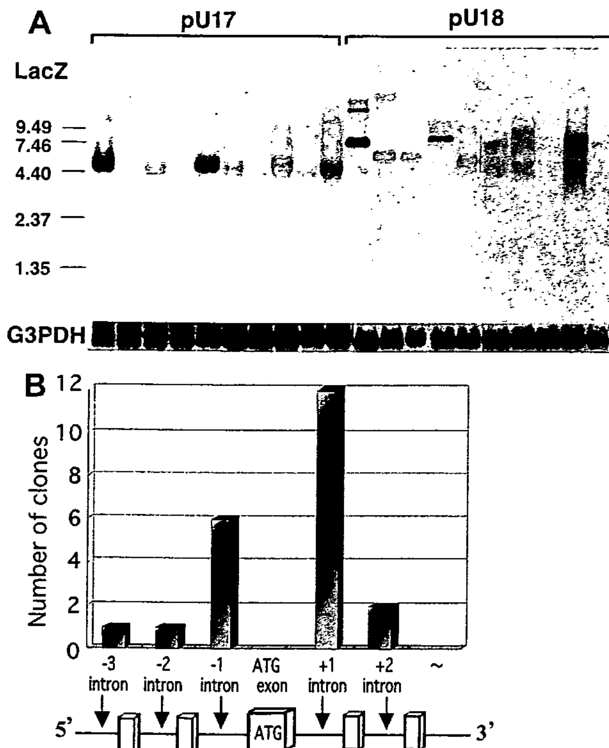

Fig. 2. (a) Northern blot analysis of trapped clones obtained using pU-17 (left) and pU-18 (right). RNA were prepared from embryonic stem (ES) clones carrying a single copy of the trap vector, and subjected to Northern blotting with a *LacZ* probe (upper) or *G3pdh* probe (lower). Size markers are shown on the left. (b) Histogram of the integration sites. Twenty-two clones in which a known gene was trapped were chosen, and the integration sites in the trapped genes were analyzed using the Celera Discovery System.

Table 2. Summary of the 5′-rapid amplification of cDNA ends (RACE) analysis results

Known	EST	Novel	ND†	Total
22 (17%)	61 (47%)	29 (22%)	17 (13%)	129 (100%)

The sequences obtained through 5′-RACE were compared to the published sequences in GenBank and the Celera Discovery System. †Not Determined: no product was obtained or part of the vector sequence (in many cases, the 5′-region of the intron sequence) was obtained.

Replacement of the βgeo gene with the EGFP gene

pU-17 carries four *lox* sites, such that the DNA sequence between *lox*71 and the other *lox* sites of *lox*P, *lox*2272 and *lox*511 can be replaced with another DNA sequence. To demonstrate the ability of pU-17 for such replacement, we performed targeted integration of the *EGFP* gene, as outlined in Figure 3. We constructed a replacement vector carrying a *lox*66-SA-*EGFP-FRT-Pgk* promoter-*Pac-FRT-loxP*-pSP73. For the targeted replacement, 20 μg of each targeting plasmid and pCAGGS-Cre were electroporated into trap ES clones in their circular forms. Because the replacement vector and trap vector in the genome carry two *lox* sites with the same spacer region (*lox*66 and *lox*P, and *lox*71 and *lox*P, respectively), it is expected that intramolecular recombination should initially occur between the two *lox* sites after co-electroporation, resulting in the production of two intermediate molecules, as shown in the middle of Figure 3. Next, the ES cells in which targeted replacement has occurred were selected in the presence of puromycin. Because the *Pac* gene in the targeting vector does not have a pA signal, random integrants should be puromycin sensitive, and only upon Cre-mediated targeted integration, the *Pac* gene fuses to the pA signal on the trap vector, thereby making the cells drug-resistant. After removal of the *Pac* gene using the Flp/*FRT* system, the *EGFP* gene is expressed under the control of the trapped promoter. This removal is achieved by mating with *CAGGS-Flp* transgenic mice.

Two trap clones were used for the replacement experiment. The Ayu17–71 clone has trapped a novel gene and the expression pattern of the *βgeo* is ubiquitous (see Fig. 4). The Ayu17–104 clone has trapped the *Shroom* gene (Hildebrand & Soriano 1999) and shows a tissue-specific expression pattern.

After electroporation, 17 and 16 colonies from Ayu17–71 and Ayu17–104, respectively, were picked, expanded and analyzed for recombination events. To confirm the 5′- and 3′-junctions, we performed PCR and Southern blot hybridization with the Pac probe, respectively. Targeted insertion should give a 1.5 kb band in the PCR and a 2.4 kb band in the Southern blotting (Fig. 3A). Figure 3(B) shows the results for Ayu17–104 subclones, of which 15 of 16 clones (94%) revealed the pattern of targeted replacement. Ayu17–71 subclones also showed a high targeting frequency of 82% (Table 3). These results demonstrate that the replacement system using poly(A) trapping functions efficiently.

Germline transmission of the replaced clones and mating with CAGGS-Flp mice

We produced chimeric mice using two replaced clones derived from each of the parental Ayu17–71 and Ayu17–104 lines, and successfully obtained germline chimeras from all the subclones used. Next, the chimeric male mice were mated with *CAGGS-Flp* transgenic mice which express the *Flp* gene ubiquitously (data not shown). In the double-positive mice for the *CAGGS-Flp* transgene and the replaced trap vector,

recombination should occur between the *FRT* sites, resulting in deletion of the PGK-pac sequence. We confirmed the recombination using tail DNA from the double-transgenic mice and Southern blotting with the *EGFP* probe. The recombined allele gives a 1.4 kb band, whereas the original allele gives a 2.8 kb band. As shown in Figure 3, the expected 1.4 kb band was obtained in the all double-transgenic mice. However, only 40–80% of the progenies from the double-transgenic mice showed the recombined pattern (data not shown), indicating that the *Flp* gene is not expressed in all the germ cells in this *CAGGS-Flp* transgenic line.

Expression pattern of the integrated EGFP gene

The *in vivo* expression pattern of the targeted integrated *EGFP* gene should be identical to that of the *βgeo* gene expression in the parental lines. First, we

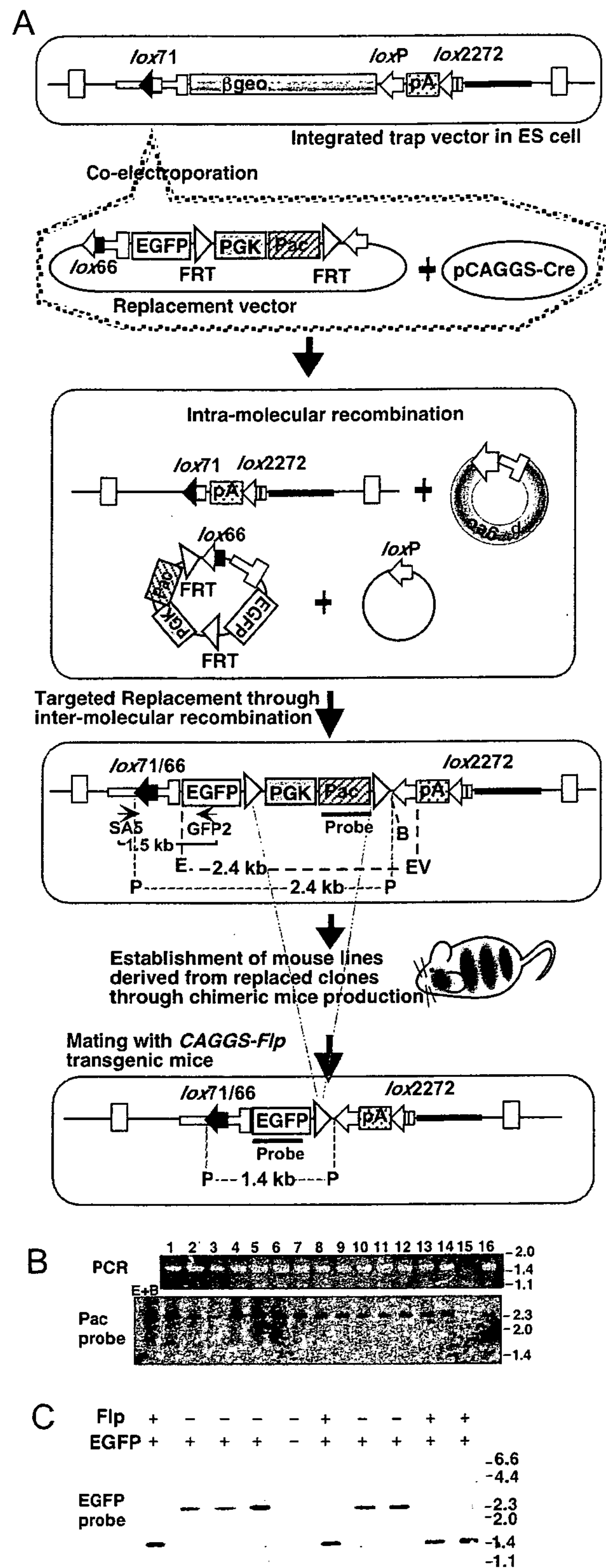

Fig. 3. Replacement of *βgeo* with *enhanced green fluorescent protein* (*EGFP*) and removal of the marker gene using the Flp/*FRT* system. (a) Scheme of the gene replacement. The structures of the integrated trap vector and targeting vector plasmid are shown in the top panel. The targeting vector p6SEFPPF carries *lox*66-SA-*EGFP-FRT-Pgk* promoter-*Pac-FRT-loxP*-pSP73. p6SEFPPF and the Cre-expression vector, pCAGGS-Cre, are co-electroporated into trap ES cells, and targeted recombinant cells are selected with puromycin. The expected intermediates produced through intermolecular recombination are shown in the second panel, followed by the allele replaced with the *EGFP* gene through Cre-mediated recombination. After establishment of a mouse line from the replaced clone, the selectable marker gene, *Pgk-Pac*, is removed by Flp/*FRT* recombination through mating with *CAGGS-Flp* transgenic mice. The recombined allele by Flp recombinase is shown in the bottom panel. The positions of the primers SA5 and GFP2, and the EGFP and Pac probes used for the Southern blotting are indicated by arrows and solid bars labeled 'probe', respectively. The expected sizes of the polymerase chain reaction (PCR) products and signals in Southern blotting are also indicated. E, *Eco*RI; EV, *Eco*RV; B, *Bam*HI; P, *Pst*I. (b) PCR and Southern blot analysis to detect targeted integration of the SA-*EGFP-FRT-Pgk-Pac-FRT* unit in subclones of Ayu17–104. Genomic DNA from 16 randomly picked subclones were subjected to PCR with SA5 and GFP2 to detect their 5′-junctions. For detection of their 3′-junctions, the genomic DNA were digested with *Eco*RV and *Eco*RI and hybridized with the Pac probe. Fifteen of 16 clones showed the expected pattern of targeted replacement, namely 1.5 kb in PCR and 2.4 kb in Southern blotting. The E + B lane represents *Eco*RI and *Bam*HI digestion, which should give 2.3 kb regardless of the integration position. (c) Detection of recombination by Flp recombinase. Genomic tail DNA of F1 offspring obtained from mating of chimeric male mice and *CAGGS-Flp* female transgenic mice were prepared and examined for the existence of the replaced allele and the *CAGGS-Flp* transgene by PCR. The PCR results are indicated at the top by a plus or minus. Next, the DNA were digested by *Pst*I and hybridized with the *EGFP* probe to detect recombination between FRT sites. Recombined alleles should give a 1.4 kb band, whereas unrecombined alleles should produce a 2.8 kb band. In all the double-transgenic mice, a 1.4 kb band is detected, indicating deletion of the PGK-Pac sequence through recombination by Flp.

Table 3. Results of targeted replacement with the *EGFP* gene

Parental cell line	Total no. of colonies	No. of colonies analyzed	No. of colonies with targeted integration	% of targeted integration
Ayu17–71	22	17	14	82
Ayu17–104	109	16	15	94

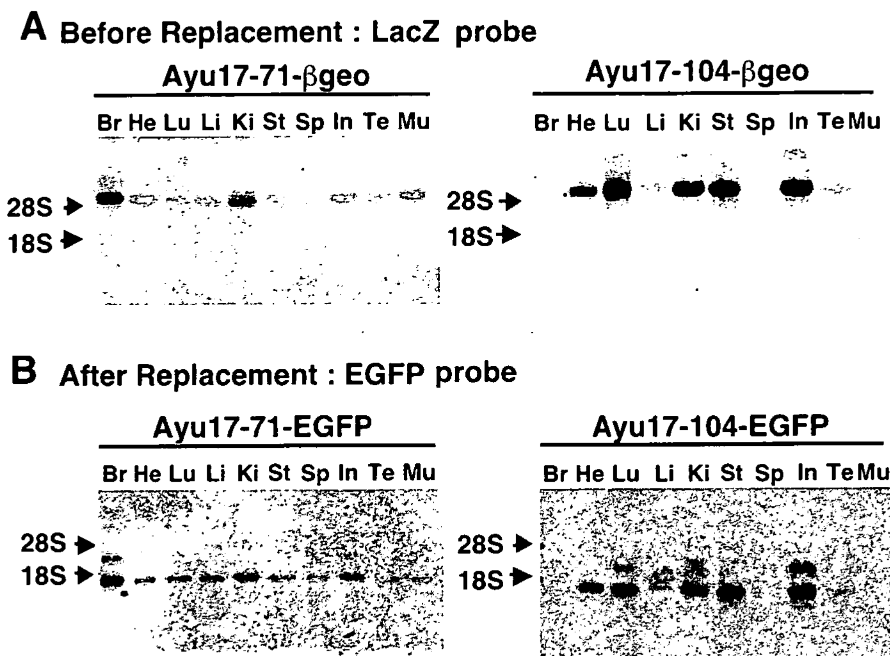

Fig. 4. Expressions of the *βgeo* and *EGFP* genes. (A) Northern blot analysis with the *LacZ* probe. Total RNA were extracted from tissues of adult hemizygous *Ayu17–71-βgeo* or *Ayu17–104-βgeo* trap mice, that had been established from parental trap clones carrying the *βgeo* gene, and subjected to Northern blotting. (B) Northern blot analysis with the *EGFP* probe. Double-positive F1 mice of the trap allele replaced with the *EGFP* gene and the *CAGGS-Flp* transgene were used. Br, brain; He, heart; Lu, lung; Li, liver; Ki, kidney; St, stomach; Sp, spleen; In, intestine; Te, testis; Mu, muscle.

examined the expression pattern by Northern blotting. Figure 4(A) shows the *βgeo* expression in heterozygous mice established from the parental trap lines before replacement, and Figure 4(B) shows the *EGFP* expression in double-positive F1 mice for the replaced trap allele and the *CAGGS-Flp* transgene. In the *Ayu17–71* line, *βgeo* expression was ubiquitous, but the brain and kidneys showed stronger expression. After replacement, the same expression pattern was observed for the *EGFP* gene. In the *Ayu17–104* line, *βgeo* expression was detected in the heart, lungs, kidneys, stomach and intestine, and the integrated *EGFP* gene showed the same expression pattern. In several tissues, extra bands of about 2.4 kb were detected. This size was identical to the size observed in the single-positive *EGFP* mice before recombination by Flp (data not shown), indicating that the recombination by Flp was not complete.

Next, we further confirmed the expression pattern at the cellular level by histological analysis with X-gal staining in the parental *βgeo* lines and immunostaining with an anti-EGFP antibody in the replaced *EGFP* lines. As shown in Figure 5, positive signals in the Purkinje cells, cerebrum and renal medulla in the *Ayu17–71* line (Fig. 5A) and the mucosa of the stomach and the glomerulus of the kidney in the *Ayu17–104* line (Fig. 5B) were detected in both staining procedures. Thus, the inserted *EGFP* gene was expressed with the same cell-type specific pattern as the original *βgeo* gene.

Discussion

Here, we have demonstrated that our exchangeable promoter trap system functioned as expected, in that the trap vector was selectively inserted into the 5′-region of endogenous genes, the *βgeo* reporter gene was easily replaced with another gene and, importantly, the inserted gene was expressed in the same pattern as the *βgeo* gene *in vivo*.

In addition to replacement of the reporter gene, many other replacements can be performed using the pU-17 trap clones, as shown in Figure 6. First, any genomic DNA or cDNA of interest can be expressed through replacement between *lox*71 and *lox*P (Fig. 6A). We can also introduce any type of mutation, such as a point or dominant-negative mutation. Second, through replacement between *lox*71 and *lox*2272, the *cre* gene can also be inserted and

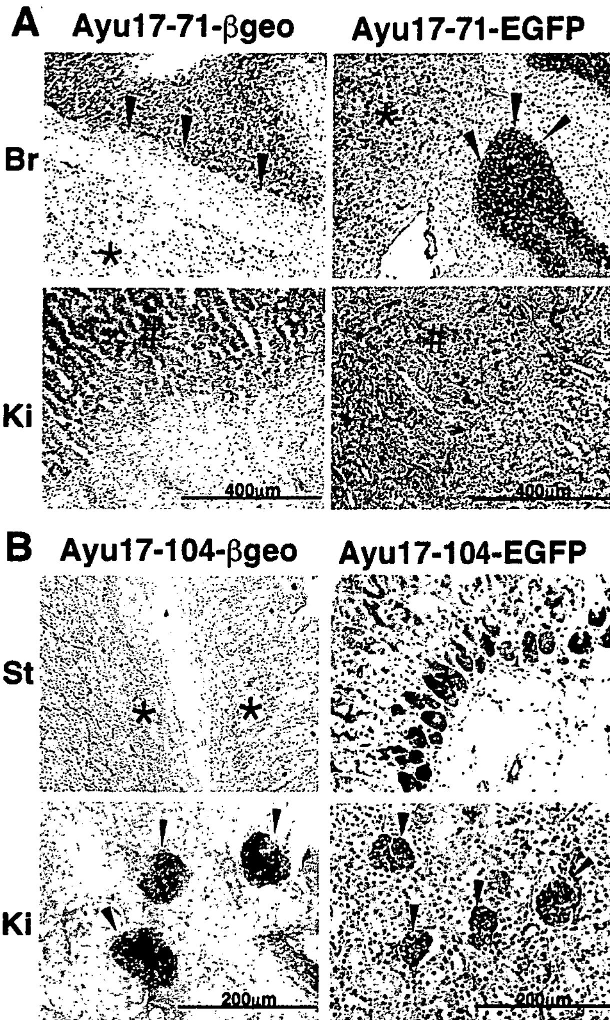

Fig. 5. Histological analysis of the expressions of the *βgeo* and *EGFP* genes. (a) The brain (Br) and kidney (Ki) from original *Ayu17–71-βgeo* and replaced *Ayu17–71-EGFP* mice were stained with X-gal (left) and an anti-EGFP antibody (right), respectively. In both staining procedures, positive signals are detected in the Purkinje cells (arrow heads), cerebrum (*) and renal medulla (#). (b) The stomach (St) and kidney (Ki) from original *Ayu17–104-βgeo* and replaced *Ayu17–104-EGFP* mice were stained with X-gal (left) and an anti-EGFP antibody (right), respectively. In both staining procedures, positive signals are detected in the mucosa of the stomach (*) and glomerulus of the kidney (arrow heads).

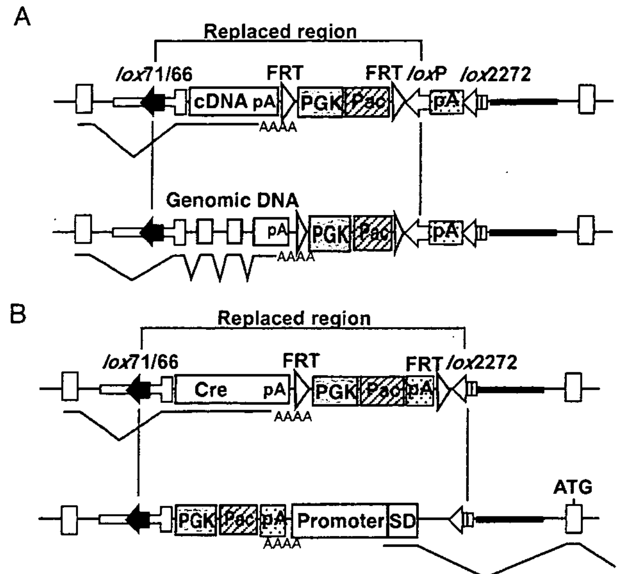

Fig. 6. Replacement patterns in trap clones with pU-17. (a) Replacement between *lox*71 and *lox*P. Any cDNA (upper) or genomic DNA (lower) of interest can be inserted and expressed under the control of the trapped promoter activity. (b) Replacement between *lox*71 and *lox*2272. The *cre* gene can also be expressed under the control of the trapped promoter activity (upper). If a promoter sequence followed by a splice donor site (SD) is inserted, it is possible to change the expression pattern of the trapped gene.

expressed (Fig. 6B, upper) as described previously (Araki *et al.* 2002). Because we can choose trap lines with the desired expression pattern by observing the *βgeo* expression, this should be convenient for the production of various Cre-mice. Third, in cases of integration of the trap vector into the 5′-region of the endogenous ATG, we can alter the expression pattern of the trapped gene by inserting an exogenous promoter (Fig. 6B, lower). By using a strong promoter in ES cells, this would be useful for 3′-RACE to determine the trapped gene when 5′-RACE fails or only produces quite a short sequence. In all cases, the selection marker gene used for replacement can be removed through mating with *CAGGS-Flp* mice.

In the pU-17 vector, we inserted in-frame stop codons in the upstream of the ATG of the *βgeo* gene for the convenience being able to express the inserted cDNA after replacement. It is not necessary to use the IRES or adjust the reading frames between the trapped gene and the inserted cDNA for this expression. Theoretically, this should reduce the colony formation efficiency, but our results revealed no significant differences in the colony formation efficiencies between vectors with and without IRES. We confirmed that the vector integration sites in pU-18 clones were completely random (data not shown), indicating that the IRES in pU-18 functioned as expected in the trap clones. We suppose that IRES-dependent translation is affected by the surrounding sequence and cannot always start efficiently. On the other hand, Bonaldo *et al.* (1998) reported that their SA-IRES-*βgeo* vector showed 6.7-fold higher colony formation efficiency than their SA-*βgeo* vector. Although the reason for this result is not clear, it would be due to the absence of the start codon of the *βgeo* in their SA-*βgeo* vector. With their vector,

therefore, trap clones become G418 resistant only when a stable and active fusion protein of the *βgeo* and trapped gene is produced from the trapped allele. We speculate that the probability of the production of active fusion protein would be much lower than that of successful trap event with pU-17.

The trap vector pU-17 showed a strong bias to integration into the 5′-region of the genes. It is well known that retroviral vectors show a tendency for integration into the 5′-end of genes (Friedrich & Soriano 1991; von Melchner *et al.* 1992), but no plasmid trap vectors have been reported to have such a tendency, except for pKC199βgeo, reported by Thomas *et al.* (2000). The vector pKC199βgeo also has an in-frame stop codon with the ATG of the *βgeo*, although they did not describe it but discussed other four possible reasons for the tendency to integrate into the 5′-end of genes. Thus, the structure of the SA carrying in-frame stop codon with the start codon of the *βgeo* would be useful for enriching integration near the 5′ end of genes.

In 64% of the trap clones, pU-17 vector was integrated into the downstream of the exon containing the start codon of the trapped gene (Fig. 2B). It is known that upstream AUG codons and open reading frames (uAUG/uORF) are common features of mRNA for mainly negative control of translation from the main AUG (Morris & Geballe 2000; Kozak 2002), and it is estimated that about half of human mRNA have uAUG/uORF (Suzuki *et al.* 2000). Leaky scanning and reinitiation mechanisms of ribosomes enable the downstream main AUG codons to be accessed by translation machinery (Kozak 2002). Although uAUG/uORF diminish translation of the main ORF, it is reported that approximately 40% of ribosomes were able to initiate twice and approximately 25% were able to initiate three times (Wang & Rothnagel 2004). Because the ORF started from endogenous AUG should act as uORF in 'downstream-integrated' trap clones, it is considered that the translation initiation of the *βgeo* would be somewhat lower level than that of the endogenous trapped gene, but enough for acquirement of G418 resistance. Whether the βgal activity in 'downstream-integrated' trap mice exactly reflect the expression pattern of the trapped genes or not can be easily tested by targeted insertion of the IRES-LacZ construct, and this analysis is now in progress.

We have performed targeted integration with more than 20 exchangeable trap clones, and in all clones except for one (Araki *et al.* 1999), we successfully obtained recombined clones at high frequencies of 80–95%. Thus, the targeted integration is highly reproducible. Our exchangeable gene trap system can overcome the limitations of conventional gene trapping and will be an ideal means for large-scale mutagenesis.

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