厚生労働科学研究費補助金（感覚器障害研究事業）
分担研究報告書

幹細胞から眼組織への分化誘導能に関する研究

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研究要旨：再生医療の材料として期待されている幹細胞の眼組織への分化誘導能を検討する目的で、本年度はマウスの各種幹細胞の生存・死・分化誘導能等の特性の検討を主要な細胞内情報伝達系であるMAPキナーゼ（ERKやSAPK/JNK）系やPAX6遺伝子を用いた眼組織への分化誘導能の観点から検討した。その結果、p38MAPキナーゼがES細胞から外胚葉系の神経細胞分化誘導に対しては抑制的に制御すること、また、PAX6遺伝子のアイソフォーム5a型が強い神経細胞分化誘導能を示すことを見い出した。これらの結果は、再生医療に求められる幹細胞の試験管内培養法や眼組織への分化誘導系を確立する上で重要な知見を提供すると考えられる。

A. 研究目的

眼組織構築に期待されているES細胞や骨髄細胞などの特性を明らかにし、将来の再生医療の基盤となる眼組織分化誘導系の開発を行う。

B. 研究方法

阻害剤や遺伝子誘導法を用いて、マウスES細胞における各種MAPキナーゼとPax6の神経細胞分化誘導に対する影響を検討した。細胞の取り扱いは、大学動物実験委員会の承認を得、そのプロトコールに従って遂行されている。

C. 研究成果

MAPキナーゼ系がES細胞から神経細胞分化誘導に抑制的役割を果たすこと,またPAX6アイソフォーム5a型が強い神経細胞分化誘導能を示すことを見い出した。

D. 考察

ショウジュウバエの眼形成にはMAPキナーゼによるEyaを含む転写因子のリン酸化が遺伝子発現の制御に関与していることが示されている。マウス眼形成においても眼形成関連の転写因子を制御している可能性が示唆された。また、哺乳動物においても幹細胞から網膜組織を構築できることが示唆された。

E. 結論

神経細胞への分化誘導に関わるシグナル伝達系の一端が解明された。また、哺乳動物においてもPAX6発現誘導系を利用した幹細胞から眼組織への分化誘導系の開発が期待される。

F. 健康危険情報

該当する危険は無し

G. 研究発表

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3) 第27回日本分子生物学会,神戸,2004年12月

H. 知的財産権の出願・登録状況

1. 特許取得　　無し
2. 実用新案登録無し

その他　本研究プロジェクトで作製された MAP キナーゼ関連分子特異抗体が、(株) 生物医学研究所から発売されている。

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分担研究報告書

視細胞の分化調節機構に関する研究

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研究要旨：　原発性・続発性に障害された神経網膜、特に網膜視細胞の再生を可能にするために、マウス網膜を用いて網膜視細胞の分化誘導に関与する調節機構の一部を明らかにし、論文を発表した。さらに、網膜視細胞の分化を細胞外微少環境と関連づけて解析し、組織内再生や細胞移植による治療法の研究に利用する。

A. 研究目的

視細胞の分化には、外的因子および内的因子の相互関係が関与するとされている。損傷網膜においては、その活性が上昇しているとされ、再生医学の見地からも興味深い外的因子 Ciliary neurotrophic factor（CNTF）が、網膜視細胞前駆細胞の内的因子に負の影響を与えるメカニズムを解析し論文を発表した。さらに、再生医学においては、未分化細胞を分化誘導する必要があるが、未分化維持シグナルとして重要視されている Notch シグナルが、網膜視細胞の分化調節機構にいかに関与しているかの解析を開始した。再生医療の見地からは、これらの情報を、組織内再生や細胞移植などの新規治療法の開発の基礎として役立てることを目的とする。

B. 研究方法

我々はマウス神経網膜を器官培養する方法を導入した。この方法ではマウス網膜の正常発生の過程をよく再現することが知られていた。我々は既にこれを利用し、外的因子である CNTF が、その下流のいずれのシグナル伝達機構を介し、視細胞の分化マーカーであるロドプシンの発現を抑制するかを解析した。その際に、我々は、周産期マウスの器官培養した網膜の、免疫染色法や *in situ* hybridization 法を用いた定性的解析法、イムノブロット法や real-time RT-PCR 法を用いた定量的解析法を確立した。また、この器官培養法を活用し、器官培養下の正常神経網膜に、直接遺伝子を導入する方法としてエレクトロポレーションによる遺伝子導入法を確立した。

これらの方法を用いて、我々は、活性型 Notch および、細胞内で Notch を不活性化しうる分子 Numb の、正常網膜器官培養への遺伝子導入とその解析を行った。さらに、Numb に対する RNAi を準備している。これを遺伝子導入により発生期の正常網膜に発現させ、Numb の網膜での Notch シグナル制御及び視細胞分化への影響を解析する予定である。

(倫理面への配慮)

動物の飼育・管理は慶應義塾大学医学部動物実験ガイドラインを遵守して行われている。

C. 研究結果

1）正常発生における、Notch、Numb の発現パターンを発生段階ごとに解析した。その結果、Notch シグナルは、視細胞の分化に先駆けて、不活性化していた。これに対し、Notch シグナルの不活性化に関与しうる Numb は同部位で同時期に、発現していた。

2）生後０日目のマウス神経網膜を器官培養し、活性型 Notch および Numb の遺伝子導入を行い、導入された細胞での細胞の運命を解析した。活性型 Notch 遺伝子が導入されると、ミューラー（グリア）細胞にも分化する細胞が増加するだけでなく、一部の介在ニューロンにも分化しており、視細胞への分化は抑制されていた。これに対し、Numb 遺伝子が導入されると、視細胞に分化する傾向にあった。

3）次に視細胞への分化には、Numb が必要かどうかを解析するために、網膜器官培養への Numb RNAi の導入をエレクトロポレーションにより行った。その結果、導入された細胞では、視細胞への分化が抑制される傾向にあった。

4）3）の方法では、最終分裂以前・以後に関わらず遺伝子が導入されていた。そこで、実際に Notch-Numb シグナルが関与する、分裂直後の細胞にのみ、遺伝子の強制発現をすべく、網膜器官培養にレトロウイルスによる遺伝子導入の系を準備中である。抗体染色により、遺伝子導入をされた細胞に限定した表現系の解析を可能にするため、導入された細胞が確実に検出可能になるような RNAi レトロウイルスベクターを開発中である。

D. 考察

胎生期の網膜（*in vivo*）で高値を示すCNTFの活性が、出生後０日目以降、急激に下降するのと同期して、視細胞の最終分化マーカーであるロドプシンの発現が開始すること、および*in vitro*においてCNTFを添加すると、ロドプシンの発現が抑制されることは従来知られていた。これがCNTF/gp130レセプター下流の主な２つのシグナル伝達経路のうち、STAT3の活性化を介して生じ、ロドプシンの上流の転写因子 *crx* の発現抑制を伴っていることは既に報告した。同時に、視細胞分化のためには、活性化STAT3以外にも解除されるべき負の調節因子や、新たに発現すべき正の調節因子の存在する可能性が示唆されていた。

今回の結果から、活性化STAT3と同様に、未分化維持の機能を持つことで知られるNotchシグナルは、最終分裂後、直ちに不活性化を受ける必要がある可能性があった。それにより、速やかに未分化維持に関わる下流の分子の発現を低下させ、タイミング良くその他の因子の影響も受け、視細胞への分化がおこる可能性があった。

これに対し、Numbは細胞の分化を促進していたが、エンドサイトーシスによるNotchシグナル抑制能を持つことが知られている。そこで、Notchシグナルの発現低下がNumbによって行われているのかどうか、また、Notchシグナルの視細胞分化への負の調節、およびNumbによる正の調節が *crx*、ロドプシンのいずれの段階で生じているのか、などさらに解析する必要がある。

E. 結論

マウス視細胞(rod)の分化マーカーである *crx* およびロドプシンの発現には、活性化 STAT3の発現低下が必要であることが明らかであった。そして、活性化STAT3は発生過程において視細胞分化に対し抑制的に働き、その発現低下は視細胞の分化の時期を決める因子の一つとして、部分的に関与していると考えられた。

STAT3は単独で最終分化マーカー、ロドプシンの発現を調節するものではなく、他の因子も共に関与する可能性が考えられた。そして、STAT3と同様、細胞の未分化維持に働き、関連して機能しうることが報告されている、Notchシグナルは視細胞への分化の運命を負に、Notch の細胞内拮抗分子Numbは正に、調節していた。組織再生のためには、未分化細胞を分化誘導して利用する必要がある。視細胞の再生医療に向けては、この機構をさらに明らかにする必要があるといえた。

F. 健康危険情報

特になし

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H. 知的財産の出願・登録状況

１．　特許取得 なし

２．　実用新案登録 なし

３．　その他 なし

厚生労働科学研究費補助金（感覚器障害研究事業）
分担研究報告書

先天ムコ多糖症の角膜混濁に対する細胞治療

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研究要旨：先天ムコ多糖症モデルマウスの角膜混濁の細胞治療を行った。正常マウスから樹立した骨髄間葉細胞株の中でβ-グルクロニダーゼ活性の高いものを選択し、モデルマウスの角膜実質内に移植した。移植細胞は少ないながら生着し、その周囲では角膜混濁の原因であるムコ多糖症の沈着が短期間で消失することが示された。

A. 研究目的

リソゾーム蓄積症は、リソゾームに局在する酵素の先天的な欠損により、消化しきれない中間代謝産物が細胞内外に蓄積する遺伝病である。最近、ゴーシェ病、ファブリー病、ムコ多糖症など一部の疾患で、骨髄移植や酵素補充療法が開発されている。しかし、眼病変、とくに角膜混濁については、効果が乏しい。我々は先に、ムコ多糖症モデルマウスを用いて、アデノウイルスベクターで欠損酵素を角膜組織内に導入する遺伝子治療を行った。今回は、骨髄間葉細胞を移植する細胞治療の有効性を検討した。

B. 実験方法

種々のマウス骨髄間葉細胞株のβ—グルクロニダーゼ活性測定および染色を行って、高値のものをドナー細胞として選定した。これにセルトラッカーで細胞表面染色を施した。ムコ多糖症（先天性β—グルクロニダーゼ欠損症）のモデルマウスである B6/MPSVII マウスの角膜実質に表層切開を行い、上記の細胞を移植した。ムコ多糖症の角膜内沈着物に対する治療効果を、準薄切切片を作成してトルイジンブルー染色行い、病理組織的に検討した。

（倫理面への配慮）本研究では、動物実験のみでヒトの材料を使った研究は計画されていない。動物実験については、国立成育医療センター研究所動物実験指針に従って実験した。

C. 実験結果

マウス骨髄間葉細胞株のうち、β—グルクロニダーゼ活性測定および染色によって、最も高値を示した 9-15C 細胞をドナー細胞に選定した。角膜実質に移植した後、２週間までは、セルトラッカーの蛍光顕微鏡下観察で、少数ながら細胞の生存が確認された。病理組織的には、角膜内に沈着したムコ多糖変性物が、移植細胞の周囲で特に消失していた。

D. 考察

ムコ多糖症では、骨髄移植療法や酵素補充療法が開発されている。これらの全身的治療法では、骨病変や内臓病変における治療効果が認められるが、中枢神経病変や角膜病変に対する効果は期待できない。前者は、血液脳関門の存在が、後者は、無血管組織という角膜組織の特殊な構造が影響していると考えられ、いずれの組織においても、局所療法が重要と考えられる。先に我々は、アデノウイルスベクターによってβ—グルクロニダーゼ遺伝子を導入し、角膜病変を治療する遺伝子治療の有用性を示した。しかし、アデノウイルスベクターでは遺伝子発現が一時期しか得られず、恒久的な発現が得られるレトロウイルスは導入効率が非常に悪い。そこで、今回はβ—グルクロニダーゼ活性の高い細胞を移植する細胞治療を試みた。その結果、細胞の生存と、その周囲のムコ多糖沈着物の消失が確認された。しかし、角膜混濁を消失させるのは、長期間かつ多数の細胞を生存させる技術の向上が必要である。これが達成できれば、早期に臨床応用できる可能性がある。

E. 結論

先天ムコ多糖症モデルマウスの角膜混濁に、β-グルクロニダーゼ活性の高い正常マウス由来骨髄間葉細胞を移植した。移植細胞は少ないながら生着し、その周囲では角膜混濁の原因であるムコ多糖沈着が消失しており、細胞治療の可能性が示された。

F. 健康危険情報

該当する危険は無し

G. 研究発表

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H. 知的財産権の出願・登録状況

1. 特許取得　　なし
2. 実用新案登録　なし
3. その他　　なし

研究成果の刊行に関する一覧表

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ORIGINAL ARTICLE

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Identification of a novel polymorphism involving a CGG repeat in the *PTCH* gene and a genome-wide screening of CGG-containing genes

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Abstract Mutations in the human homologue of the *Drosophila patched* gene (*PTCH*) are responsible for the hereditary disorder called nevoid basal cell carcinoma syndrome (NBCCS). *PTCH* has a CGG triplet repeat located 4 bp upstream of the first methionine codon. Here we report a novel polymorphism involving the number of the CGG-repeat. The major allele (86.3%) contained a repeat size of seven, whereas the minor allele contained eight. No significant difference in the distributions of genotypes was observed between normal and NBCCS individuals. However, when the repeat was inserted between a heterologous promoter and the luciferase gene, the longer repeats tended to induce higher luciferase activities, suggesting that the repeat length potentially affects the levels of gene expression. A genome-wide screening revealed that 68 and 146 genes contained a CGG/CCG repeat in the coding region and in the 5'-untranslated region (5'-UTR), respectively. None of the genes had this repeat in 3'-UTR. Interestingly, the number of genes with a CGG repeat in the 5'-UTR was significantly higher than that with a CCG repeat in the 5'-UTR. The localization of a CGG/CCG repeat in *PTCH* is quite unique in that only four other genes have been found in which the repeat is localized up to 4 bp upstream of the first methionine.

Keywords PTCH · Nevoid basal cell carcinoma syndrome · Gorlin syndrome · Polymorphism · Triplet repeat

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Introduction

Mutations in the *PTCH* gene are responsible for the hereditary disorder called nevoid basal cell carcinoma syndrome (NBCCS; MIM# 109400) (Hahn et al., 1996; Johnson et al., 1996). NBCCS, also called Gorlin syndrome, is an autosomal dominant neurocutaneous disorder characterized by developmental abnormalities and tumorigenesis such as palmar and plantar pits, jaw cysts, calcification of the falx cerebri, skeletal anomalies, basal cell carcinoma, ovarian fibroma, and medulloblastoma (Gorlin, 1987). *PTCH* (MIM # 601309) is a human homologue of the *Drosophila* segment polarity gene *patched*. It has been mapped to 9q22.3-q31 and consists of 23 exons encoding a protein with 1,447 amino acid residues. The PTCH protein is a receptor for a secreted molecule Sonic hedgehog and has twelve transmembrane domains. At least two forms of PTCH protein are known to exist, reflecting the use of alternative exon 1a versus 1b (Hahn et al., 1996; Wicking et al. 1997a). Mutations in exon 1b have not been investigated so far due to, at least in part, the extreme GC-rich sequence (Wicking et al. 1997a; Fujii et al. 2003a). In the course of analyzing mutations in exon 1b, using a new set of primers and a PCR condition, we discovered a novel polymorphism involving a CGG trinucleotide repeat immediately upstream of the first in-frame methionine codon. We compared allele frequencies between healthy individuals and NBCCS patients. We also investigated the effect of the repeat length on the gene expression using a heterologous reporter gene. In addition, the results of a genome-wide screening of CGG/CCG-containing genes are demonstrated.

Materials and methods

DNA samples

After informed consent was obtained from 51 healthy, unrelated individuals and 14 patients with NBCCS, total genomic DNAs were isolated from peripheral leukocytes by the standard phenol/

chloroform extraction method. Patients were diagnosed as having NBCCS according to the clinical criteria (Kimonis et al. 1997). All studies were approved by the local ethnic committee. Among 14 patients with NBCCS, *PTCH* mutations were found in 11. Some of the mutations have already been reported (Fujii et al. 1999, Fujii et al. 2003a, Fujii et al. 2003b) and some will be reported elsewhere.

Polymerase chain reaction and sequencing

The genomic region of *PTCH* including the 5'-untranslated region (5'-UTR) and exon 1b was amplified by using the forward primer, 5'-CGCGCAATGTGGCAATGGAA-3', and the reverse primer, 5'-AGAGGAGGGAAGAGAAAGTG-3'. The polymerase chain reaction (PCR) was carried out in a 20 µl reaction volume by using LA Taq with GC Buffer (TaKaRa) according to the manufacturer's instruction. PCR was run for 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 3 min on a Program Temp Control System PC-800 (ASTEC, Fukuoka, Japan). Both the sense and antisense strands of the PCR products were directly sequenced by using the same primers as described above. PCR products purified by a QIAquick PCR Purification Kit (QIAGEN) were used as the template DNA for cycle sequencing with a CEQ DTCS Quick-Start Kit (Beckman Coulter). Sequencing analysis was performed on a CEQ 8000 Genetic Analysis System (Beckman Coulter) according to the manufacturer's instructions.

Plasmid construction

Luciferase constructs containing the sequence of *PTCH* 5'-UTR were generated by a PCR-mediated method described previously (Imai et al. 1991) using pGV-P2 (Wako Chemicals, Osaka, Japan) as a template. The authenticity of all constructs was confirmed by sequencing.

Luciferase assay

The human embryonic kidney cell line 293 growing on six-well culture plates were cotransfected using Effectene reagent (QIAGEN) with 0.5 µg of luciferase plasmid and 0.5 µg of pCMVβGal. The cells were harvested at 24 h after the transfection and used for a luciferase assay. Luciferase activities were measured as described previously and normalized for transfection efficiency based on β-galactosidase activities (Shikama et al. 2001).

Real-time quantitative RT-PCR

Total RNA was extracted from the transfected cells described above using TRIzol reagent (Invitrogen). One-step RT-PCR was performed with a 7700 ABI PRISM Sequence Detector System (Perkin Elmer-Applied Biosystems) using primers 5'-TCTGGATCTACTGGTCTGCCTAA-3' and 5'-GCGCACTTTGAATCTTGTAATCCTG-3'. To normalize the expression of luciferase, the *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) housekeeping gene was also amplified, using primers 5'-GAAGGTGAAGGTCGGAGT-3' and 5'-GAAGATGGTGATGGGATTTC-3'. Fluorogenic probes 5'-CAAATCATTCCGGATACTGC-3' and 5'-CAAGCTTCCCGTTCTCAGCC-3' carrying 5' 6-carboxy-fluorescein as a reporter dye and 3' 6-carboxy-tetramethyl-rhodamine as a quencher dye were used to detect the PCR product of luciferase and *GAPDH*, respectively. In every experiment, *GAPDH* was amplified using a series of dilutions of a known amount of the standard RNA supplied by Perkin Elmer to prepare a standard curve.

Computational screen

Human mRNA sequences that contain more than seven repeats of CGG were downloaded from NCBI nucleotide databases using the search program termed as "Search for short, nearly exact sequences" with $(CGG)_7$ as a query (http://www.ncbi.nlm.nih.gov/BLAST/). A full screening of the genes of interest was confirmed because genes with exact matches (bit score 42) were followed by the genes with partial matches (bit score less than 42). Genes for unidentified coding sequences were excluded from further study.

Statistical analysis

Genotype distributions and allele frequencies of CGG repeat numbers were compared between cases and controls by means of the χ^2 test. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated by Wolf's method.

Results and discussion

The *PTCH* gene has two alternative first exons—exon 1a and exon 1b (Fig. 1A). Exon 1b contains the first in-frame methionine codon, while exon 1a is a noncoding exon. We noticed a CGG trinucleotide repeat located 4 bp upstream of the first methionine codon. Although exon 1b is a coding exon, mutations in this exon have not been reported. In the course of analyzing mutations in exon 1b using samples from NBCCS individuals that do not have a mutation elsewhere in *PTCH*, we discovered a novel polymorphism involving the CGG trinucleotide repeat (Fig. 1B). The major allele contained

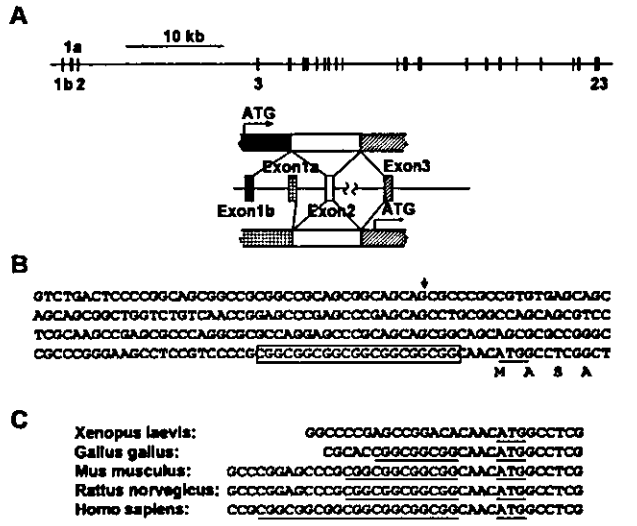

Fig. 1 A Genomic organization of human *PTCH*. The *PTCH* locus based on the sequence AL161729 is shown at the *top*. Two cDNA sequences, GenBank U43148 and U59464, are generated by alternative splicing using exon 1a and 1b, respectively, as schematically depicted at the *bottom*. B Nucleotide sequence of the human *PTCH* gene including 5'-UTR and exon 1b. The first methionine codon is *underlined*. Polymorphic CGG repeat is *boxed*. The putative transcription start site is indicated by an *arrowhead*. C Nucleotide sequence alignment of the *PTCH* genes. CGG repeat and the first methionine codon are underlined

seven repeats of CGG, while the minor one contained eight (Table 1). As far as we examined, we did not find repeat numbers other than seven or eight. The repeat is conserved among vertebrates, since chicken, mouse, and rat *PTCH* contain four or five repeats of CGG (Fig. 1C). However, the repeat has not been found in Xenopus *PTCH*, indicating it is not conserved in amphibians.

Abnormal expansion of the CGG triplet repeat in the 5'-UTR of the *fragile X mental retardation-1* (*FMR1*) gene is responsible for fragile X syndrome, in which the repeat is abnormally hypermethylated, resulting in the silence of the *FMR1* (reviewed by Jin et al. 2000). Since CGG repeat in *PTCH* is immediately upstream of the first in-frame methionine codon, the repeat number may influence the efficiency of translation as well as of transcription. To address this issue, various lengths of $(CGG)_nCAAC$ were subcloned into the luciferase plasmid pGV-P2 between the SV40 promoter and the coding sequence for luciferase (Fig. 2A), and luciferase assays were performed. Luciferase activities gradually increased with the number of CGG repeats, at least within the range we examined. The highest level of luciferase activity was obtained when cells were transfected with the plasmid pGV-$(CGG)_{19}CG(CGG)_6$, which was generated by chance during PCR reaction (Fig. 2B). These results suggest that individuals with $(CGG)_8/(CGG)_8$ have higher levels of PTCH protein expression than those with $(CGG)_7/(CGG)_7$. This is contradictory to the case of *FMR1*. However, it should be noted that in fragile X syndrome, the repeat is massively expanded over 230, and the repeat is located more than 50 bp upstream of the first methionine codon.

To address the question of whether the difference in luciferase activity is transcriptional or translational, the levels of luciferase RNA expression were quantified by a real-time RT-PCR. As shown in Fig. 2C, in contrast to the activities of luciferase, no significant difference in luciferase transcription was observed. Moreover, unexpectedly, the cells transfected with the plasmid pGV-$(CGG)_{19}CG(CGG)_6$ expressed significantly lower levels of luciferase RNA. Therefore, the increase in luciferase activities with the expansion of the CGG repeat is due to the increased efficiency of translation.

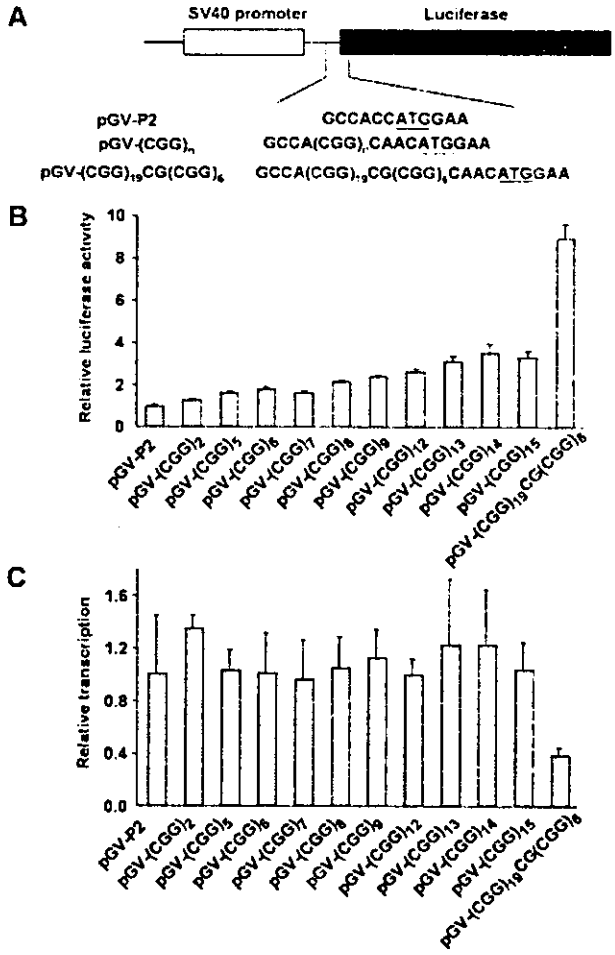

Fig. 2 A Schematic depiction of reporter gene constructs used for a luciferase assay. Nucleotide sequences inserted between SV40 promoter and the luciferase gene are indicated at the *bottom*. The first methionine codon of the luciferase gene is *underlined*. **B** The effect of the repeat length on luciferase activities; 293 cells transfected with plasmids indicated at the *bottom* were harvested 24 h after the transfection and subjected to a luciferase assay. **C** The effect of the repeat length on luciferase transcriptions. Total RNA was extracted from 293 cells transfected with plasmids indicated at the *bottom* and subjected to a real-time RT-PCR. Luciferase transcriptions were normalized by those of *GAPDH*

Table 1 Genotype data of a $(CGG)_n$ on the *PTCH* gene

	Controls	NBCCS
Major allele (repeat number: 7) [%]	88 [86.3]	25 [89.3]
Minor allele (repeat number: 8) [%]	14 [13.7]	3 [10.7]
Total	102 [100.00]	28 [100.00]
Major homozygous [%]	39 [76.5]	12 [85.7]
Heterozygous [%]	10 [19.6]	1 [7.1]
Minor homozygous [%]	2 [3.9]	1 [7.1]
Total	51 [100.0]	14 [100.0]
χ^2 [*P*]		
Genotype frequency (2x3 table)	1.38 [0.50]	
Allele frequency (major versus minor)	0.18 [0.68]	
Major homozygous versus others	0.56 [0.46]	
Minor homozygous versus others	0.26 [0.61]	
Odds ratio [95% CI]		
Major homozygous versus others	1.85 [0.36–9.43]	
Minor homozygous versus others	1.88 [0.16–22.44]	
Major allele versus minor allele	0.75 [0.20–2.83]	

Table 2 CGG/CCG-containing genes. *UTR* untranslated region

	$(CGG)_n$	$(CCG)_n$	Total
CDS	39	29	68
5' UTR	95[a]	51[a]	146
Total	134	80	214

Genes with a repeat of seven or more CGG/CCG were downloaded from NCBI nucleotide databases:
[a]The number of CGG-containing genes are significantly higher than CCG-containing genes (χ^2 test, $P=0.00027$)

Table 3 $(CGG)_n/(CCG)_n$-containing genes in which the triplet repeat is located immediately upstream of the first in-frame methionine codon

Accession no.[a]	Gene[b]	Nucleotides between $(CGG)_n/(CCG)_n$ and ATG	CGG or CCG	n
BC029158	Clone MGC:34313 IMAGE:5198758	CCCCCGGGGC	CCG	10
NM_025075	Hypothetical protein FLJ23445	CGCACGCC	CCG	8
BC015930	Clone MGC:8881 IMAGE:3920963	CGGGGGCC	CCG	8
NM_013396	Ubiquitin specific protease 25 (USP25)	CGGGGGCC	CCG	8
NM_013272	Solute carrier family 21 (organic anion transporter), member 11 (SLC21A11)	GGGAAGG	CGG	8
NM_005360	Transcription factor C-MAF (c-maf)	CAGGAGA	CGG	7
NM_004699	DNA segment on chromosome X (unique) 9928 expressed sequence (DXS9928E) (XAP-5)	CTGCC	CCG	9
NM_000264	PTCH	CAAC	CGG	7
NM_145296	TSLC1-like 2 (TSLL2)	CACC	CGG	7
NM_002958	RYK receptor-like tyrosine kinase (RYK)	CCC	CGG	7
NM_017811	Ubiquitin-conjugating enzyme E2R 2 (UBE2R2)	CG	CCG	7
NM_173054	Reelin (RELN)	C	CGG	8–10[c]

[a]One representative accession number for one gene
[b]Genes that have less than ten nucleotides between triplet repeat and the first methionine
[c]Repeat number varies depending on deposited sequences

The distributions of genotypes that we observed in NBCCS patients and controls did not differ from the expected frequencies under the assumption of Hardy-Weinberg equilibrium (data not shown), nor were significant associations with NBCCS observed (Table 1). Thus far, no genotype-phenotype correlation between the position of mutations and major clinical features of NBCCS is evident (Wicking et al. 1997b). Since developmental defects associated with the disorder are most likely due to haploinsufficiency, and the repeat length potentially alter the expression levels of PTCH, the repeat number may have an effect on the severity of the disease. It would also be interesting to examine the association of the repeat number with sporadic or non-inherited basal cell carcinoma or medulloblastoma, since PTCH acts as a tumor suppressor in these tumors (reviewed by Hunter 1997).

In order to find other genes with CGG repeats, we next performed a genome-wide screening of CGG/CCG-containing genes from NCBI nucleotide databases. A total of 214 genes having seven or more of the repeat number were downloaded. A complete list of the CGG/CCG-containing genes can be obtained from our Web site, http://genetics.nch.go.jp/supplements.htm. Of those 214 genes, 146 (68.2%) contained the repeat in the 5'-UTR (Table 2). Interestingly, significantly more genes have CGG repeats than CCG repeats (65.1% versus 34.9%, $P=0.00027$). More significantly, none of the downloaded genes contained repeats in the 3'-UTR. The genes containing CGG/CCG repeats in close proximity to their first methionine codons are listed in Table 3. Only five genes including *PTCH* have intervening sequences of up to 4 bp between $(CGG)_n/(CCG)_n$ and ATG. In this regard, *PTCH* is quite unique in terms of the location of the repeat. Considering our results, polymorphisms of the repeat number that might exist in these genes potentially affect their expression levels.

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Identification of novel direct transcriptional targets of glucocorticoid receptor

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Transcription of the genes *Granzyme A* (*GZMA*), *FK506 binding protein 51* (*FKBP5*), and *Down syndrome critical region gene 1* (*DSCR1*) is upregulated in leukemic cells upon treatment with glucocorticoids (GCs). Several lines of evidence suggest that these genes are implicated in GC-induced apoptosis upstream of the Bcl-2 family of proteins. These genes were upregulated by GC even in the presence of an inhibitor of protein synthesis, cycloheximide, indicating that they are direct target genes of glucocorticoid receptors. *DSCR1* is reported to have four isoforms, each of which has a distinct first exon, E1–E4. Among these isoforms, the one with E1 was selectively upregulated by GC. *GZMA* and *FKBP5* have a cluster of putative glucocorticoid response elements (GREs) in introns 1 and 2, respectively, that was identified to be responsible for the response to GC. They were composed of one complete (A/T)G(A/T)(A/T)C(A/T) sequence surrounded by two incomplete (A/T)G(A/T)(A/T)C(A/T) sequences separated by one to four nucleotides. *DSCR1*, however, did not have a functional GRE upstream or downstream of exon 1. These studies may lead to improved therapeutic uses of GCs in leukemia and lymphoma based upon the expression of these GC target genes.
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Introduction

Glucocorticoids (GCs) are known to be potent immunosuppressive, antiallergic, and anti-inflammatory drugs. They exert their effects on target cells by binding to an intracellular glucocorticoid receptor (GR). The ability of GCs to induce apoptosis and cell cycle arrest in lymphoid cells has resulted in their widespread use as chemotherapeutic agents for various leukemias, lymphomas, and multiple myelomas, although the precise mechanism of their actions is yet to be elucidated.[1,2] Glucocorticoid receptor (GR) is a member of the nuclear hormone receptor superfamily localized in the cytoplasm. Inactive GR is bound to a large protein complex that includes heat shock protein 90 (Hsp90). When GC binds to GR, Hsp90 dissociates and the GC/GR complex translocates to the nucleus where it binds to specific palindromic sequences, termed glucocorticoid response elements (GREs), resulting in the transcriptional upregulation of various genes.[3]

We previously investigated transcriptional changes during GC-induced apoptosis in GC-sensitive human pre-B leukemia 697 cells harboring the t(1;19) chromosomal translocation[4] using oligonucleotide microarrays.[5] Among 93 genes induced by a synthetic GC, dexamethasone (DEX), were *Granzyme A* (*GZMA*), and two other genes encoding calcineurin inhibitors, *FK506 binding protein 51* (*FKBP5*) and *Down syndrome critical region gene 1* (*DSCR1*). Granzymes are serine proteases and are packaged in cytotoxic granules of CTL and NK cells, together with the pore-forming protein perforin. The concerted action of these molecules induces apoptosis of target cells, such as infected cells or transformed tumor cells.[6,7] *DSCR1*, a gene located on chromosome 21, is highly expressed in fetal brain and is suggested to have a role in brain development. The product of *DSCR1* interacts with the catalytic A subunit of calcineurin and inhibits its phosphatase activity, and thus is also called modulatory calcineurin-interacting protein 1 (MCIP1).[8] *FKBP5* encodes FK506 binding protein of 51 kDa, which is known as an immunophillin, and also regulates the inhibition of calcineurin.[9,10] These three genes are suggested to mediate GC-induced apoptosis in lymphoid cells by the following findings. First, the granzyme inhibitor 3,4-dichloroisocoumarin has been reported to inhibit DEX-induced apoptosis of 697 cells.[11] Second, the activation of calcineurin protects T cells from GC-induced apoptosis.[12,13] Moreover, using a variety of pre-B leukemic cells, the expression of the latter two genes evoked by GC and the induction of apoptosis were found to be closely correlated.[5] However, the induction of these genes does not necessarily mean that they are direct transcriptional targets, because they may be regulated via a secondary effect induced by the primary targets of GC. In addition, GR regulates transcription by binding to and inhibiting or enhancing the function of other transcription factors such as AP-1, NF-κB, and the STAT family of transcription factors, which can change the transcriptional profile.[14,15] Therefore, we investigated the genomic organization of these genes and whether they are direct target genes of GR.

Materials and methods

Cells and reagents

Pre-B human leukemia 697 cells were routinely grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 50 U/ml of penicillin, and 0.1 mg/ml of streptomycin. HeLa and COS-7 cells were maintained in DMEM with the same supplements. DEX and cycloheximide (CHX) were purchased from Sigma.

Plasmid construction

To generate pGRE-Luc, the oligonucleotides 5′-TCGATCAGAA CACTGTGTTCTGA-3′ and 5′-TCGATCAGAACACAGTGTTCT GA-3′ were annealed and subcloned into the *Xho*I site of pGV-P2 (Wako Chemicals, Osaka, Japan). Genomic sequences corresponding to human *GZMA*, *FKBP5*, and *DSCR1* were amplified by PCR with appropriate primers using the Expand

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High Fidelity PCR system (Roche Diagnostics) and subcloned into a luciferase vector, pGV-B2 or pGV-P2 (Wako Chemicals). Reporter constructs with mutated sequences were generated by the PCR-based method described previously.[16] Detailed information on the primers used for the PCR is available at Leukemia's website. All the constructs were verified by DNA sequencing.

Analysis of gene expression by RT-PCR

To analyze the isoform-specific regulation of *DSCR1*, 500 ng of total RNA extracted from 697 cells was reverse-transcribed and cDNA was amplified by PCR using a forward primer specific to *DSCR1* exon 1 or 4, and a reverse primer for exon 5. Logarithmically amplifying PCR product was subjected to agarose gel electrophoresis. For real-time RT-PCR, one-step RT-PCR was performed using a 7700 ABI PRISM Sequence Detector System (Perkin Elmer-Applied Biosystems). Fluorogenic probes carrying 5′ 6-carboxy-fluorescein as a reporter dye and 3′ 6-carboxy-tetramethyl-rhodamine as a quencher dye were used to detect the PCR product. In every experiment, the *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene was amplified using a series of dilutions of a known amount of the standard RNA supplied by Perkin Elmer to prepare a standard curve. Data analysis was performed as described[17] with slight modifications. Detailed information on the primers used for the PCR is available at Leukemia's website.

Western blot analysis

Western blot analysis was performed as described previously[5] using goat anti-FKBP51 polyclonal antibody (F-13, Santa Cruz) followed by horseradish peroxidase-conjugated donkey anti-goat immunoglobulins (Santa Cruz). The proteins were visualized using an enhanced chemiluminescence method (Amersham).

Transfection and luciferase assay

HeLa cells growing in six-well plates were transfected with 750 ng of the reporter gene plasmid along with 500 ng of pCMVβGal and 750 ng of an expression plasmid for rat GR, p6RGR[18] (a gift from Keith Yamamoto), using Effectene reagent (Qiagen). The medium was replaced with DMEM without phenol red (Invitrogen) with 10% charcoal-treated fetal calf serum just before the lipofection. The cells were harvested at 16 h after transfection and used for a luciferase assay. Luciferase activities were measured as described previously and normalized for transfection efficiency based on β-galactosidase activities.[19]

Electrophoretic mobility shift assay

COS-7 cells were transfected with p6RGR and 10^{-6} M of DEX was added. Nuclear extract was prepared 48 h after transfection by three cycles of freezing and thawing in cell resuspension buffer (40 mM HEPES-KOH, pH 7.9, 0.4 M KCl, 1 mM dithiothreitol, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1% aprotinin) and incubated with an end-labeled double-stranded oligonucleotide probe: FKBP5 GRE2 5′-AGTAACA CAATGTACAGGTTTGTAGCATTG-3′; GZMA GRE3 5′-TGG GAGAATCCAAGAACATCTGGTGCAGGA-3′; GZMA GRE4 5′-TGTGTTTAGTTCTCACTGTTCC-3′. The reaction was performed in 15 μl of binding buffer (4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 0.05 mg/ml poly(dI-dC)) for 20 min at room temperature. The supershift analysis was performed by including anti-GR polyclonal antibody (Affinity Bioreagents) in binding reactions. Samples were fractioned on a nondenaturing 6% polyacrylamide gel and visualized by autoradiography.

Results

Three candidate target genes are upregulated by GC in the absence of de novo *protein synthesis*

We and others have identified *GZMA*, *FKBP5*, and *DSCR1* as GC-responsive genes using microarray technology.[5,20–23] However, it is likely that some of the GC-induced genes identified are regulated because of secondary effects induced by the primary targets of GC. To rule out this possibility, we analyzed the effect of GC in the presence and absence of an inhibitor of protein synthesis, CHX. Evidently, protein synthesis was indeed shut down by CHX as the level of FKBP5 protein declined in cells growing in the presence of CHX even after DEX treatment, in contrast to the experiment without CHX (Figure 1a). Hence, direct target genes of GC will be transcriptionally activated, but because of the inhibition of protein synthesis, the GC-induced proteins will not be synthesized and will not induce secondary

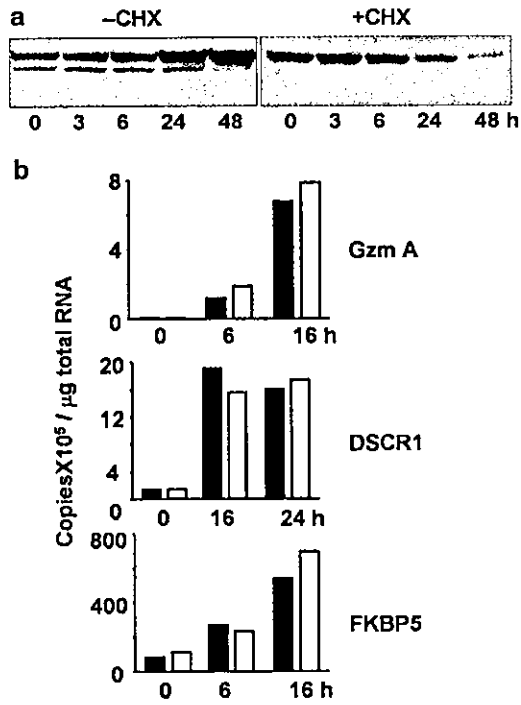

Figure 1 Three genes are directly upregulated by DEX. (a) Cell lysates were obtained from 697 cells treated with 10^{-6} M of DEX for the periods indicated and subjected to Western blotting to detect FKBP5. In some experiments (indicated by +CHX), 10 μg/ml of CHX was added at 16 h prior to DEX treatment. (b) Transactivation of the three genes by DEX is CHX-insensitive. 697 cells growing in the presence (open columns) or absence (closed columns) of CHX for 16 h were treated with DEX for the periods indicated and then total RNA was extracted. cDNA encoding the three genes was amplified by RT-PCR and analyzed with a 7700 ABI PRISM Sequence Detector System. The expression of the genes normalized to that of *GAPDH* is shown. The analysis was performed in triplicate.

or indirect targets in this experimental system. As shown in Figure 1b, these three genes were induced significantly irrespective of the presence of CHX as assessed by real-time RT-PCR, suggesting that their expression was induced directly by GC without the need for protein synthesis.

The exon 1 variant of DSCR1 *is responsive to GC*

The human *DSCR1* gene was reported to express four variant mRNAs with each of four alternative exons (exons 1–4) incorporated selectively at the 5′ terminus of the expressed transcript.[8] The majority of these transcripts were identified to represent isoforms that include exon 1 or 4 (Figure 2a) and the other two were not detectable in human fetal and adult tissues by Northern analysis.[8] Exon 1 and 4 isoforms use distinct promoters and their transcription is differentially regulated. For example, expression of the exon 4 isoform, but not the exon 1 isoform, is selectively increased by calcineurin activity, creating a negative feedback circuit.[24] To address the issue of which variant is responsible for the upregulation of *DSCR1* by GC, we constructed RT-PCR primers that specifically recognize these isoforms (Figure 2a). As shown in Figure 2b, transcription of the exon 1 isoform (Exon1-5) is upregulated by DEX showing a similar time course as total *DSCR1* expression (Exon7-7). However, the exon 4 isoform (Exon4-5) was barely detectable in 697 cells and its expression level was not increased after the treatment with DEX. The exon 4 isoform was detected in the heart and kidney in which this variant was reported to be significantly expressed,[8] demonstrating that these primers were capable of efficiently recognizing the exon 4 isoform (Figure 2c). Collectively, GCs were demonstrated to upregulate the exon 1 isoform of *DSCR1*. Since *DSCR1* spliced from exons 1 and 4 would generate DSCR1 proteins with distinct N-terminus, it would be interesting to see if there is a difference in calcineurin inhibition between these two protein variants.

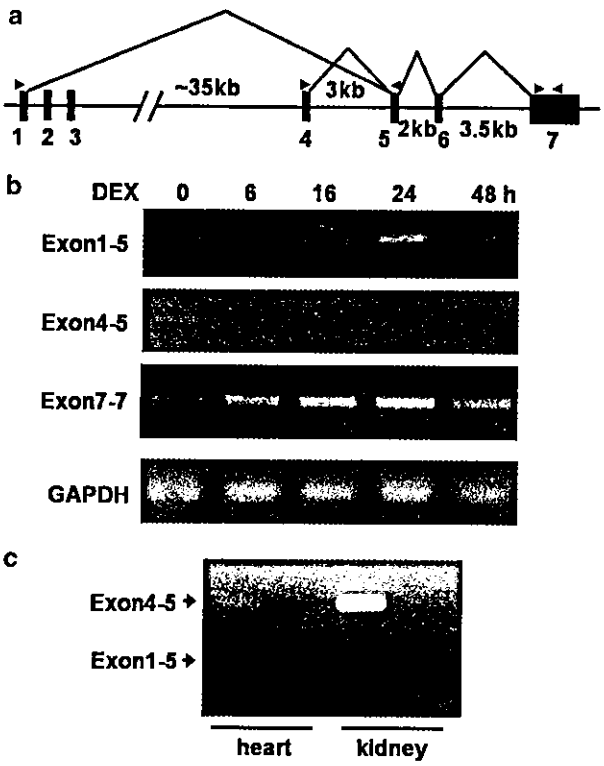

Figure 2 Variant-specific transcriptional regulation of DSCR1. (a) Genomic organization of the human *DSCR1* gene, indicating four alternative initial exons (1–4) and three exons common to all forms of *DSCR1* mRNA (5–7). Locations of two forward primers in initial exons and a reverse primer in exon 5, as well as a pair of primers in exon 7 to detect the total amount of *DSCR1* mRNA are indicated by arrowheads. (b) Semiquantitative RT-PCR analysis to detect *DSCR1*. 697 cells were treated with 10^{-6} M of DEX for the time periods indicated and total RNA was extracted for RT-PCR. Locations of the primers used for RT-PCR are indicated on the left. The *GAPDH* gene was amplified as an internal control. (c) RT-PCR analysis using adult human tissues. RT-PCR was performed similarly except that RNAs from human tissues were used as templates.

Intronic regions of GZMA *and* FKBP5 *are required for a transcriptional response to GC*

Having shown that the expression of the three genes was induced directly by GC, we amplified the promoter and intron fragments from human genomic DNA by PCR and subcloned them into a luciferase vector in order to further substantiate the role of the transcriptional activation of these genes. Despite extensive analysis of the reporter gene, no sequence responsive to GC was identified in the region around exon 1 for *DSCR1* (at least not up to ~2.2 kb and down to ~2.0 kb from exon 1), although a couple of candidate GREs were found in this region (data not shown). These results suggest that the functional GRE is located at a position distant from exon 1 or the transactivation of *DSCR1* is mediated by a nonclassical mechanism.

The *GZMA* promoter was also unresponsive to GC at least up to ~2.3 kb from exon 1 (Figure 3a, pGV-GzmPro). However, when an ~2.7 kb fragment of intron 1 was subcloned into a reporter vector, it was significantly upregulated by DEX (pGV-GzmInt). The degree of induction was more than two-fold that with pGV-GRE in which the consensus 1 × GRE (5′-AGAA CACTGTGTT-3′) was subcloned. To narrow down the region reactive to GC, the 2.7 kb segment was divided into three and various deletion mutants were tested for the response to DEX. As shown in Figure 3b, the response to DEX was completely eliminated when the middle segment of ~1 kb was deleted from the construct (pGV-GzmIntA and pGV-GzmIntC) indicating the presence of a GC-responsive element in this fragment. It was also suggested that the downstream sequence in intron 1 has an inhibitory effect on GC-induced transactivation since the constructs lacking this segment showed significantly higher induction (pGV-GzmIntAB and pGV-GzmIntB). In fact, inspection of the DNA sequence in this middle segment revealed the presence of four candidate GREs that partially match the previously reported consensus sequence (numbered GRE1 to GRE4 as shown in Figure 3c). We therefore introduced a series of nucleotide substitutions at these sites to map the actual GREs. When either GRE3 or GRE4 was mutated, DEX-mediated transactivation was markedly compromised down to less than 10-fold (pGV-GzmIntB3, pGV-GzmIntB4). In addition, their combined mutations virtually eliminated DEX-mediated transactivation (pGV-GzmIntB234, pGV-GzmIntB1234). In contrast, reporter constructs with a mutation of either GRE1 or GRE2 still showed more than 20-fold activation by DEX (pGV-GzmIntB1, pGV-GzmIntB2). These results imply that GRE3 and GRE4 are functional GREs, although GRE1 and GRE2 may have an auxiliary role.

The *FKBP5* gene was similarly examined for the presence of a functional GRE. Again, the promoter sequence of ~2 kb did not respond to the treatment with DEX (Figure 4a, pGV-FKBPPro).

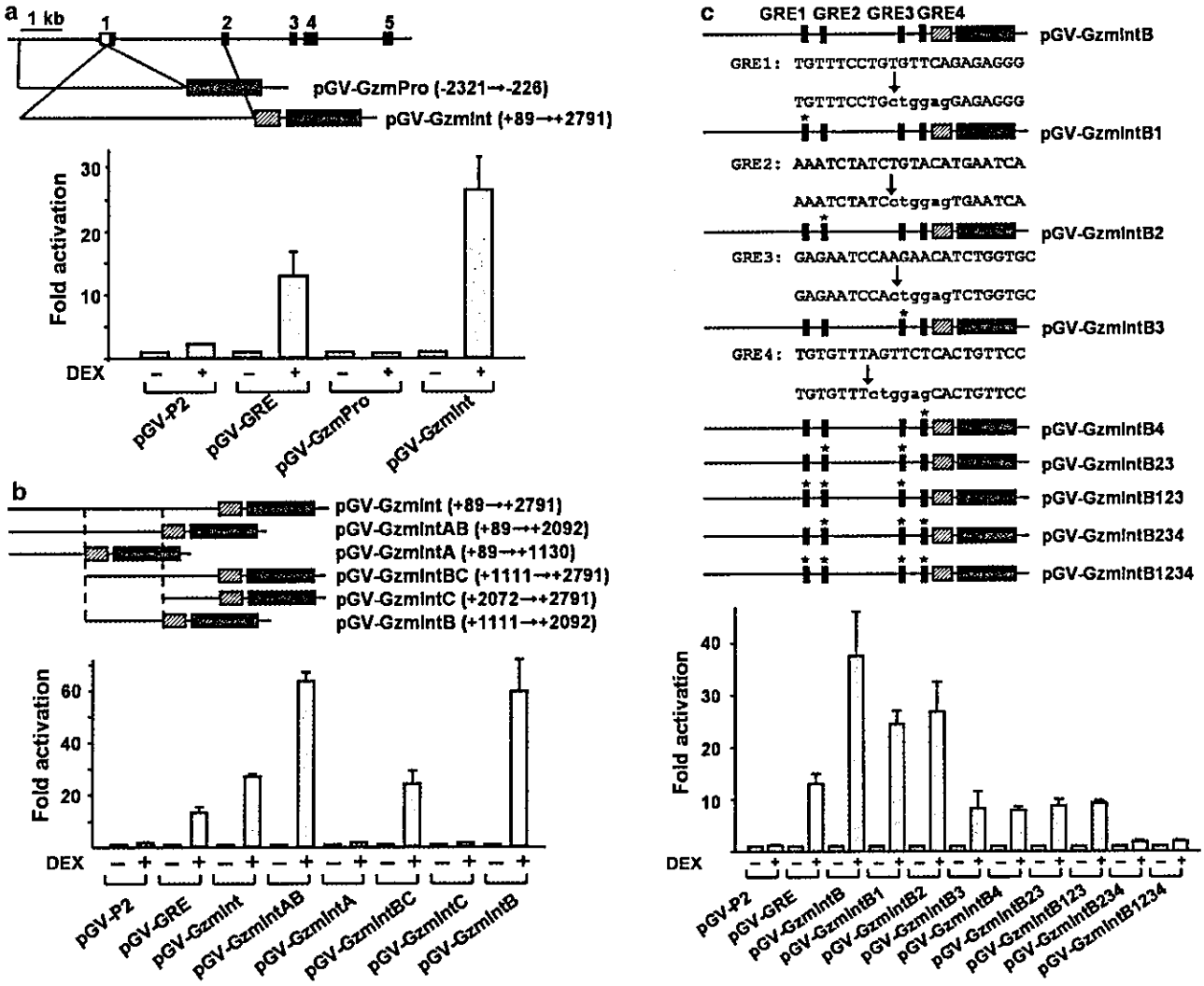

Figure 3 Regulation of the *GZMA* intron by GC. (a) The genomic organization of the human *GZMA* gene and reporter gene constructs used for luciferase assays are schematically depicted at the top. Open and filled boxes indicate noncoding and coding exons, respectively. Striped and gray boxes indicate DNA sequences encoding the SV40 promoter and luciferase, respectively. Numbers in parentheses denote nucleotide positions relative to the translational initiation site based on the genomic sequence, AC091977. Results of transient transfection assays of GZMA-luciferase reporter plasmids are shown in the bottom panel. HeLa cells were transfected with the indicated reporter gene plasmid along with pCMVβGal and p6RGR. The medium was replaced with DMEM containing 10% charcoal-treated fetal calf serum without phenol red (Invitrogen) just before the lipofection. Cells grown in the presence or absence of 10^{-6} M DEX were harvested at 16 h after transfection and used for a luciferase assay. Luciferase activities normalized for β-galactosidase activities were expressed as fold activation. Data are representative of three experiments with similar results. (b) The middle part of intron 1 is GC-responsive. To narrow down the region reactive to DEX, intron 1 was divided into three and subcloned into the reporter vector as depicted in the upper panel. Luciferase assays were performed and expressed as described in (a). (c) Identification of GRE in intron 1. Candidate GRE sequences were mutated as indicated by a PCR-based method. Lowercase letters indicate mutated nucleotides. Thick vertical bars represent the location of candidate GREs. Mutated GREs are marked by asterisks. Luciferase assays were performed and the results were expressed as described in (a).

Since introns 1 and 2 are ~46 and ~5.5 kb long, respectively, we inspected the vicinity of exons 1 and 2 for the presence of possible GREs. Four candidate GREs found in this region were subcloned into a luciferase vector and subjected to reporter gene analyses (Figure 4a). The luciferase constructs containing two candidate GREs in intron 2 (pGV-FKBPIntC) significantly responded to DEX to the same degree as pGV-GRE, whereas the constructs containing the other two candidate GREs in intron 1 were unresponsive to DEX (pGV-FKBPIntA and pGV-FKBPIntB). When the downstream GRE of pGV-FKBPIntC, but not the upstream one, was mutated, the response was virtually eliminated, suggesting that the downstream GRE is functional (Figure 4b, pGV-FKBPIntCmt2).

GR can bind in vitro *to an oligonucleotide probe representing the* GZMA *and* FKBP5 *gene region*

To determine whether the GR protein can bind to the sequences corresponding to the GREs in *GZMA* and *FKBP5* that proved to be functional in reporter gene assays, electrophoretic mobility shift assays were performed. As shown in Figure 5a, when nuclear extracts were obtained from COS-7 cells transiently transfected with expression plasmid for GR and incubated with a radiolabeled DNA probe containing a GRE sequence in the intronic region, a complex with a shift in gel mobility was detected (lanes 1, 4, and 7). When anti-GR antibody was included in binding reactions, the bands detected in the lanes

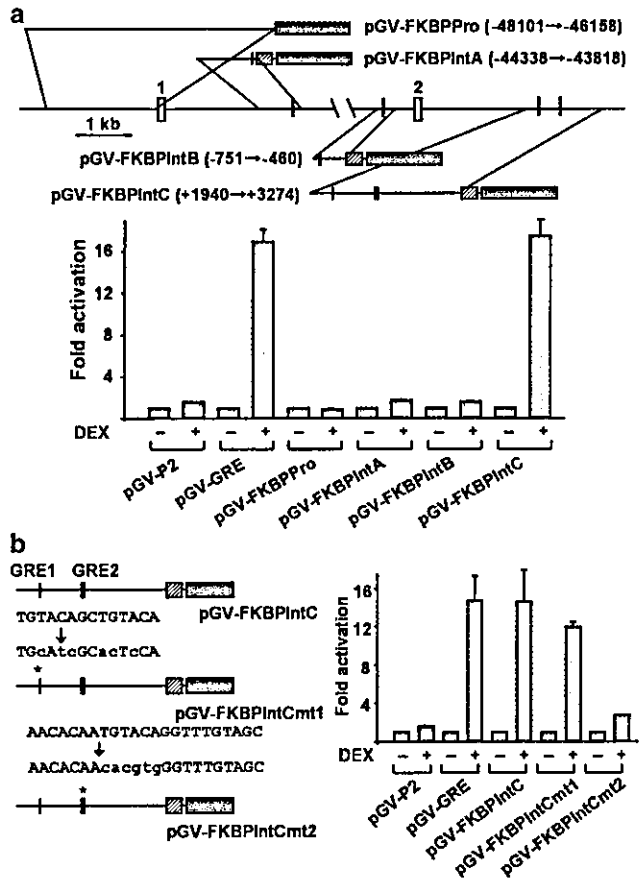

Figure 4 Regulation of the *FKBP5* intron by GC. (a) The genomic organization of the human *FKBP5* gene and reporter gene constructs used for luciferase assays are schematically depicted at the top. Open and filled boxes indicate a noncoding and a coding exon, respectively. Exons 1 and 2 are separated by a sequence ~46 kb long. Numbers in parentheses denote nucleotide positions relative to the translational initiation site in exon 2 based on the genomic sequence, AL033519 and AL590400. Thick vertical bars represent the location of candidate GREs. Luciferase assays were performed and the results were expressed as described in Figure 3. (b) Identification of GRE in intron 2. Candidate GRE sequences were mutated as indicated by a PCR-based method. Lowercase letters indicate mutated nucleotides. Mutated GREs are marked by asterisks. Luciferase assays were performed and the results were expressed as described in Figure 3.

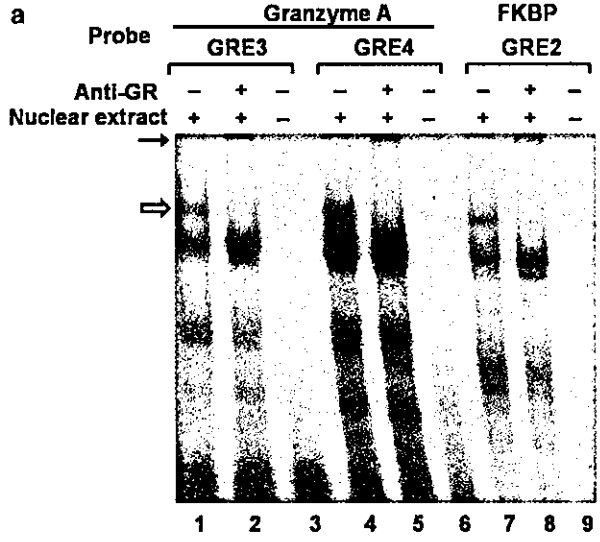

Figure 5 Electrophoretic mobility shift assays and alignment of GC-reactive sequences. (a) GR can bind *in vitro* to an oligonucleotide probe representing the *GZMA* and *FKBP5* gene region. Nuclear extracts were obtained from COS-7 cells transiently transfected with expression plasmid for GR and incubated with ^{32}P-labeled oligonucleotide DNA probes containing putative GRE sequences with or without polyclonal antibody against GR as indicated. DNA–protein complexes were size-fractionated in a nondenaturing polyacrylamide gel and detected by autoradiography. The shifted and supershifted complexes are indicated by open and closed arrows, respectively. (b) DNA sequences demonstrated to be reactive to GC are aligned. The consensus motif deduced from the alignment is indicated at the bottom. Nucleotides that fit and do not fit into the consensus motif are indicated by solid and broken underlines, respectively. Lowercase letters indicate nucleotides that separate 5-bp consensus sequences. W indicates the nucleotide A or T.

mentioned above were replaced by supershifted bands (lanes 2, 5, and 8), demonstrating the specificity of the complex formation. These results indicate that GR binds to the GRE sequences in the *GZMA* and *FKBP5* genes *in vitro*, which were identified by reporter gene assays.

Discussion

Although a number of reports have been published on the genome-wide screening of GC-induced genes,[5,20–23,25] to our knowledge, no reports have dealt with the molecular mechanism of how screened genes are transactivated by GC. Among the genes screened in our previous study, we focused on *GZMA*, *DSCR1*, and *FKBP5*, which have reportedly been relevant to GC-induced apoptosis, and found that all three are direct targets of GC. In the *GZMA* and *FKBP5* genes, GC-responsive intronic sequences to which GR can bind *in vitro* have been determined. None of the promoter sequence we analyzed responded to GC. However, this is not unexpected because a number of transcription factors, for example p53, transactivate target genes by binding to intronic sequences.[26–28] Recently, it was reported that ~400 bp of promoter sequence of human *FKBP5* exhibited upregulation by progestin in human breast cancer cells.[29] In contrast, our reporter construct, which contained this region, was unresponsive to GC, although the receptors for GC and progestin are known to recognize a sequence that is similar, if not the same.[30] Given their findings that no classical progestin-responsive element was identified in this region and the activity of luciferase was stimulated only 2.5-fold by a progesterone analog, much weaker than >10-fold effect observed in our study, it is speculated that progestin mainly regulates the