有効なsiRNAの報告がある．ここでは，我々が作製したC型肝炎ウイルス（HCV）に対するsiRNAについて[5]紹介する．

2. siRNAターゲットサイトの選択

HCV遺伝子は9600塩基からなるプラス1本鎖RNAで，5'と3' 非翻訳領域（UTR）に挟まれたORFからなる．5'側の341塩基のUTRは複雑なRNA構造のIRES（internal ribosome entry site）（一部コアタンパクコード領域に及ぶ）を含み，HCV RNAはキャップ非依存的にこの5' IRESにより翻訳される．3' UTRにはポリU配列と98塩基からなる3'X領域が存在している．ORFは5'からC, E1, E2, p7の構造蛋白，NS2, NS3, NS4A, NS5A, NS5Bの非構造蛋白を含む3010のアミノ酸からなる1本の大きなポリプロテインをコードしている（**図1上**）．

HCVは1本鎖RNAウイルスであるがゆえ，プルーフリーディング機能がなく，ウイルス複製時に特にORF領域においてRNAポリメラーゼの読み違いによる変異を起こし易い．HCV遺伝子が同定されて以来，様々な遺伝子型が報告されてきたが，現在では分子進化学的に遺伝的に距離をもつ6つの遺伝子型に分類，整理されている．また，同一個体内においても遺伝子配列の異なったウイルス集団が存在してquasispeciesと呼ばれている．

Quasispeciesの問題から，もしsiRNAにその配列上ターゲットサイトとのミスマッチ変異が生じた場合，特にその変異部位が19 ntのうち5'から9-13塩基目付近であると，たとえ1塩基でも大きく切断効率を下げる場合があることが報告されている[14]．実際にHIVでsiRNAの効果がHIVに生じた点変異で著しく減弱すること報告されている[15]．そこで我々はHCVの遺伝子型に関わらず92-98%配列が保存されている5'UTR IRESにsiRNAのターゲットを絞ってデザインした（**図1下**）．siRNAの至適配列については，一定の法則がわかっている．siRNAがRISCに取り込まれる際にアンチセンス鎖がとりこまれるために，アンチセンス鎖の5‘末端の内部エネルギーが低いことが望ましく5‘末端がAまたはUであるなどが重要とされる[16][17][18]．

3. HCVに対するsiRNAの効果

HCVは通常の培養細胞には感染せず，感染培養細胞がないことが，HCV研究の大きな妨げとなっていたが，1999年

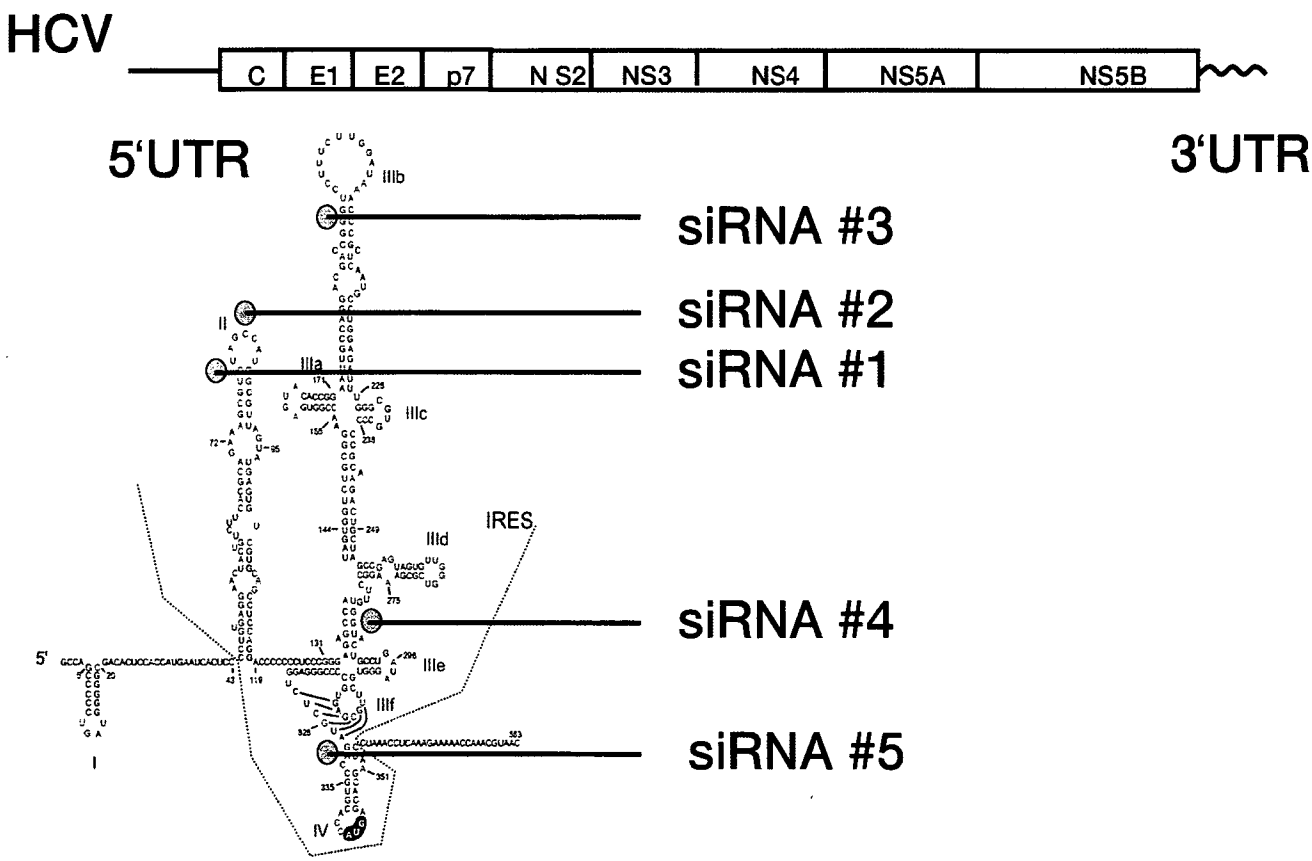

図1 （上）HCVの遺伝子構造と
（下）HCV 5'UTR IRESのRNAの2次構造とsiRNAのターゲット部位

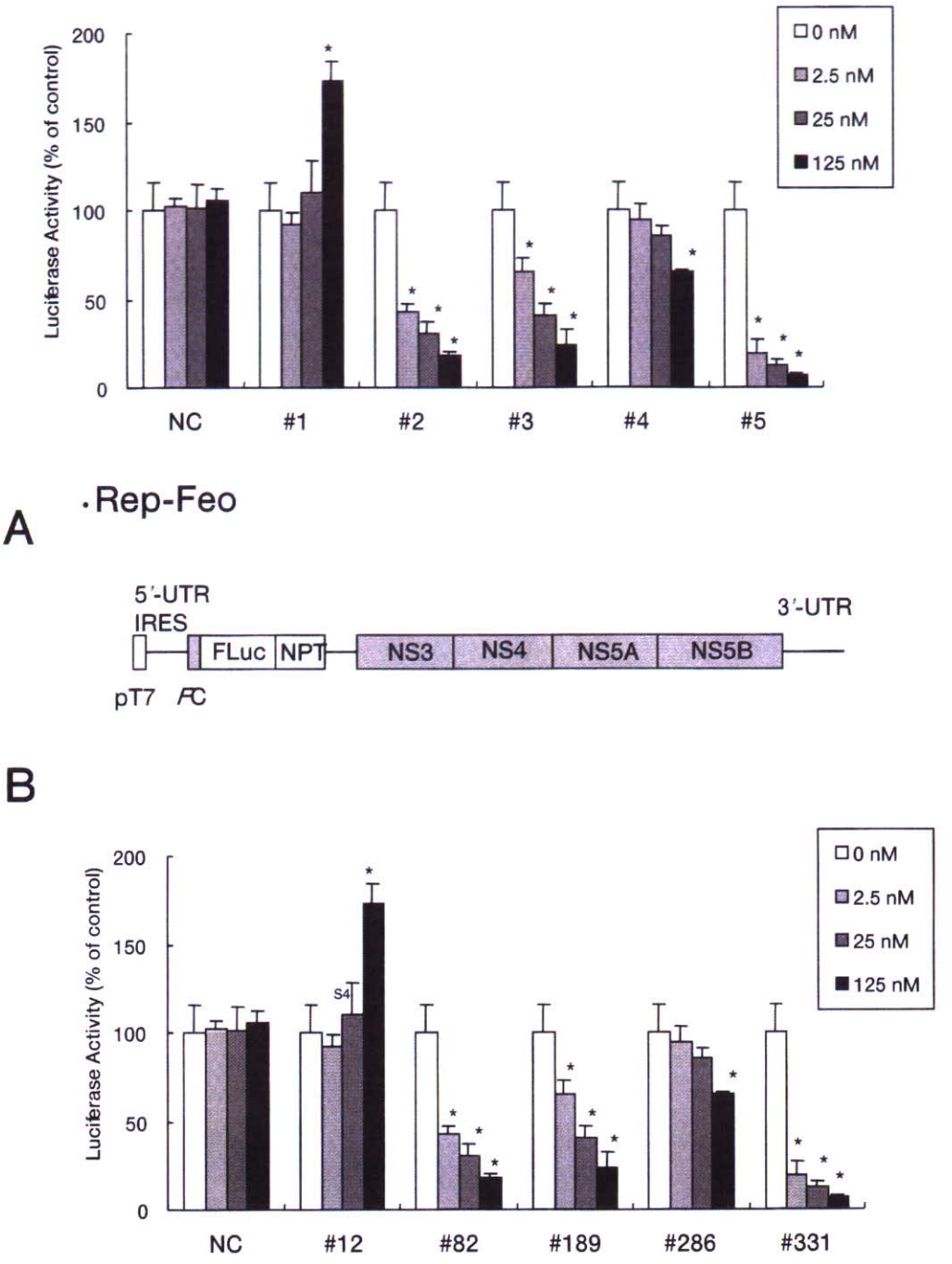

図 2　合成 siRNA の HCV レプリコン（Rep-Feo）へのルシフェラーゼ活性抑制効果（文献 5 から転載）

に Bartenschlagar らによりヒト肝細胞癌株 Huh-7 細胞を用いて HCV の自己増殖を可能にした HCV レプリコンが報告された．HCV ゲノムの構造蛋白をコードする部分をネオマイシン耐性遺伝子に置換した構造で，ヒト肝細胞癌株 Huh-7 細胞に導入して，HCV の複製機構を介して獲得したネオマイシン耐性によって安定増殖クローンが選択され，さらにレポーター遺伝子としてネオマイシン耐性遺伝子にルシフェラーゼ遺伝子を融合させて，HCV 遺伝子複製効率をルシフェラーゼ活性によって簡便に評価できるようになった．

図 2 に我々の 5′ UTR IRES に対してデザインした siRNA の効果を示す．上記 HCV レプリコン（Rep-Feo）システムにおいて siRNA331 が最も有効に発現を抑制した．コントロールに比較して 125 nM の siRNA 濃度では 97% のルシフェラーゼ活性の抑制が達せられ，2.5 nM の非常に低濃度 siRNA でも約 80% の抑制が見られた．この結果

はHCV レプリコン RNA のノザンブロットや非構造タンパクのウエスタンブロットでも確かめられた（**図3**）．このsiRNA の効果は従来の機能性核酸試薬であるアンチセンスオリゴDNA，リボザイムと効果の比較しても圧倒的に低濃度でより高い抑制活性であった．

また，ウイルス遺伝子そのものを標的とするのではなく，ウイルス増殖に必要な宿主側の内在性遺伝子を標的にする方法も考えられている．HIV 感染における TSG101[19] や NF κ B p65[20] サブユニットなどを siRNA で発現を抑制し，HIV ウイルス増殖を抑制したとの報告もある．

さらに，CD4 や CCR5 などの HIV-1 感染におけるリンパ球側に内在するウイルス受容体を標的としてその発現を抑制する方法も成果があり注目されている[21]．CD34+ 造血幹細胞に CCR5 に対する siRNA をレンチウイルスで安定発現させたところ，正常に分化して in vitro でマクロファージに in vivo で T リンパ球になり，その両者ともに HIV ウイルスに抵抗性になったとの報告がされ，今後の臨床応用に期待が持たれている[22]．

4. HCV に対する siRNA 発現ウイルスベクターの in vivo 効果

siRNA をウイルスベクターで細胞内に発現するためには siRNA 発現 DNA ベクターの作製が必要である．我々は Pol III 系のプロモーターであるヒト U6 プロモーターの下流に

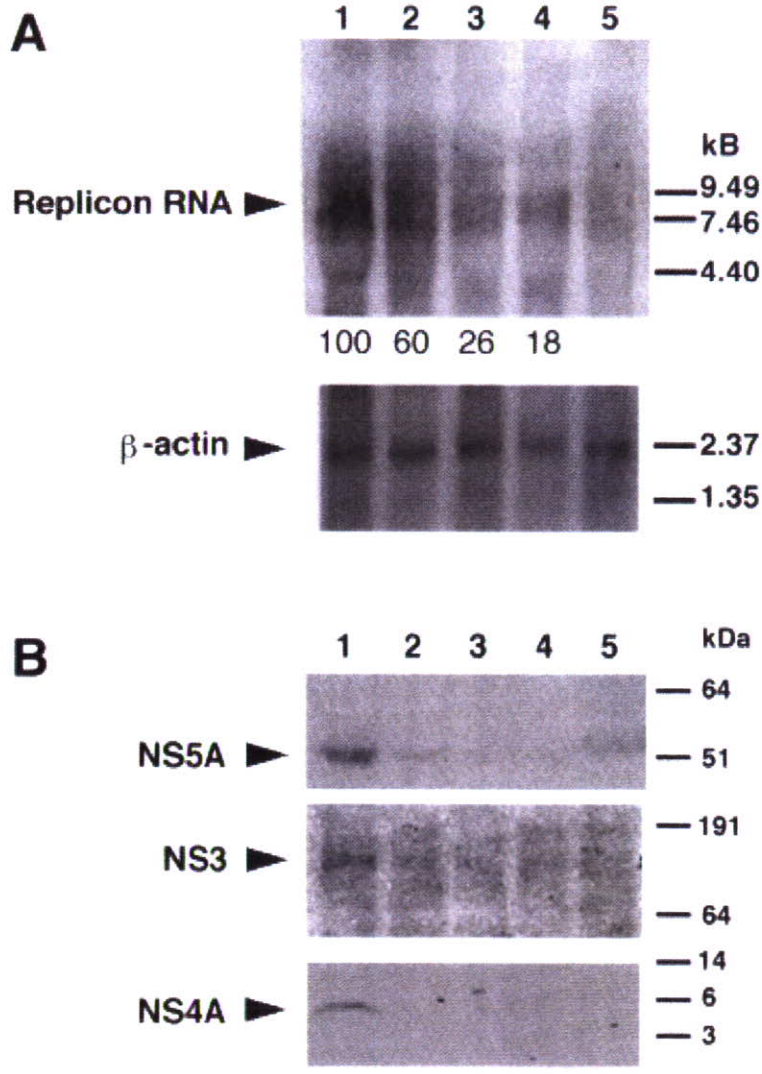

図3 siRNA331 の HCV レプリコン RNA, 非構造タンパクへの発現抑制効果（文献5から転載）
(A) レプリコン配列全長に対するプローブを用いたノザンブロット
(B) NS5B に対するプローブや抗体を用いたウエスタンブロット
lane 1, mock; lane 2 siRNA 331 2.5 nM; lane 2 siRNA 331 25 nM; lane 2 siRNA 331 125 nM; no transfection

ステムループタイプのsiRNA発現ベクター配列を挿入してその効果を検索した．ステムループタイプでは複製に際してセンスやアンチセンス配列に高頻度に変異が入ることが知られており，それを防ぐため複数のミスマッチ変異をセンス鎖に導入した[23]．これによって，HCVゲノム切断効率は下がらず，より安定なsiRNA発現ベクターが完成した．さらにこれをアデノウイルスベクターに導入することによって，HCVレプリコンのルシフェラーゼ活性を測定感度以下にすることに成功し，これを用いて肝臓にHCVゲノムが発現するトランスジェニックマウスにおいて，in vivoでのHCV遺伝子発現抑制に成功している（論文投稿中）．さらに，現在，サルでのC型肝炎モデルにおいてその効果を検証中である．

5. siRNAのin vivoへのデリバリー

siRNAは細胞質でRISCに取り込まれて切断活性を発揮することより，siRNAのデリバリーは細胞膜さえ越えればよく，遺伝子治療によく使われる発現DNAベクターのように核にアクセスする必要がない．McCaffrey[24]らはマウスの尾静脈から10-50（gの合成siRNAを体重の5-10％の大量のPBS溶液で5-7秒の短時間で注入するハイドロダイナミックス導入法で，マウスの肝細胞にsiRNAの導入に成功した．この方法によって腎臓，脾臓，肺，すい臓にも有効なsiRNAの導入が可能である[25]．このハイドロダイナミックス導入法で導入されたFas[26]やcaspase 8[27]に対する合成siRNAで，マウスに誘発された激症肝炎による死亡率を低下させとの報告がされた．このハイドロダイナミックス導入法をそのまま臨床応用することは難しいが，siRNAが*in vivo*で有効に作用することを示した重要な報告である．最近，siRNAのセンス鎖の3‘末端にコレステロールを結合させることにより，通常の速度の静脈注射でも肝臓と腸管への導入が可能でありことが示された[28]．その他，肝臓への有効なカチオニックリポソームベクターが次々と開発されており[29][30]，期待できる．

長期の抑制効果にはウイルスベクターが必要となる．ステムループタイプアピン型siRNA発現ベクターコンストラクトをアデノウイルス[31]やレンチウイルス[32]，レトロウイルス[33]，アデノ随伴ウイルス[34]などのウイルスベクターに組み込んで作製したsiRNA発現ウイルスベクターを用いて，in vivoの細胞へのsiRNA導入の報告が次々とされている．特に最近開発されたアデノ随伴ウイルスの新しい血清型8型（AAV-8）は非常に高い遺伝子導入効率があり期待されている[35,36]．

6. おわりに

siRNAによる臨床応用にはsiRNAのデリバリーとoff-targetが大きな問題点であろう．肝臓はsiRNAのデリバリーの面では最も良い臓器の1つと考えられ，特にウイルス性肝炎はsiRNAのターゲットの疾患として今後の発展に大いに期待が持たれる．

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Gene therapy of virus replication with RNAi

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RNA interference (RNAi) is the process of sequence-specific, post-tanscriptional gene silencing initiated by double-stranded RNA (dsRNA). The short interference RNA (siRNA) cleaves target RNA even in mammalian cells without adverse effects of long dsRNA such as an interferon response, and works much more efficiently than antisense oligonucleotide and ribozyme. The clinical application of siRNA has been tried especially for the viral diseases. There are still important problems for application of gene therapy including off-target effect and gene delivery of siRNA, but a rapid progress can be expected because of the extremely high efficiency of siRNA.

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RESEARCH ARTICLE

Screening of siRNA target sequences by using fragmentized DNA

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Abstract

Background RNA interference (RNAi) has become a powerful tool in silencing target genes in various organisms. In mammals, RNAi can be induced by using short interfering RNA (siRNA). The efficacy of inducing RNAi in mammalian cells by using siRNA depends very much on the selection of the target sequences.

Methods We developed an siRNA target sequence selection system by first constructing parallel-type siRNA expression vector libraries carrying siRNA expression fragments originating from fragmentized target genes, and then using a group selection system. For a model system, we constructed parallel-type siRNA expression vector libraries against DsRed and GFP reporter genes.

Results We carried out the first screening of groups containing more than 100 random siRNA expression plasmids in total for each target gene, and successfully obtained target sequences with very strong efficacy. Furthermore, we also obtained some clones that express dsRNAs of various lengths that might induce cytotoxicity.

Conclusions This system should allow us to perform screening for powerful target sequences, by including all possible target sequences for any gene, even without knowing the whole sequence of the target gene in advance. At the same time, target sequences that should be avoided due to cytotoxicity can be identified.

Keywords siRNA target sequences; enzymatic; fragmentized DNA; RNAi; RNA interference; screening

Introduction

RNA interference (RNAi), which was discovered first in *Caenorhabditis elegans* [1], is a post-transcriptional gene-silencing phenomenon induced by double-stranded RNA (dsRNA). This phenomenon seems to be conserved in evolutionarily diverse organisms [2–5]. In RNAi, dsRNAs are cleaved by Dicer, a member of the RNase III family, into small interfering RNAs (siRNAs) of 21–23 nucleotides (nts) in length [6,7]. siRNAs in turn induce the degradation of target mRNA, and, finally, the expression of the target gene is suppressed. Although it was initially difficult to induce RNAi in mammalian cells due to the interferon response induced by dsRNAs, it is now known that RNAi in mammalian cells can be induced by 21–23-nt RNAs with 2- or 3-nt 3′ overhangs [8,9] or by longer dsRNAs with predetermined mutations within the sense strand [10,11]. Furthermore, various groups, including our own, have developed siRNA expression vectors by using pol III promoters such as the U6, H1 and $tRNA^{Val}$ promoters [12–19].

The efficacy of siRNA for target gene knockdown depends very much on the target sequences selected. Therefore, choosing an appropriate target sequence is one of the key obstacles that need to be overcome for the application of RNAi in both experimental and therapeutic fields. Although algorithms and computer programs for target sequence selection have been developed, here we demonstrate a different approach for determining appropriate target sequences; that is, we construct parallel-type siRNA expression vector [12] libraries by using fragmentized DNA from the target gene. A similar method for constructing siRNA vectors by using fragmentized DNA was reported while the present work was in preparation [20], but because the siRNA expression vectors in that study are hairpin-type ones, they contain perfect matching sense and antisense sequences that form a palindromic DNA structure, which makes them difficult to construct and causes a reduction in the stability of the vector [11]. Compared with hairpin constructs, the alternative parallel-type system provides a simpler method to construct high-stability siRNA expression vector libraries [11]. We demonstrate here that, by using this system, we were able to successfully determine siRNA target sequences that produced high RNAi activities for both DsRed and GFP from their respective pools of parallel-type siRNA expression vectors containing DNA fragments of the target genes.

Materials and methods

Preparation of siRNA expression sequences from fragmentized target genes

The target gene sequences (DsRed and GFP) were amplified from the coding sequences of each gene (ptBid-DsRed; constructed previously in our laboratory, and pHygEGFP; Clontech, Mount View, CA, USA) by polymerase chain reaction (PCR), purified by using phenol/chloroform extraction and ethanol precipitation and eluted into 20 μl TE. To obtain siRNA expression sequences, approximately 1 μg of the amplified target gene DNA was fragmentized by using 0.1 to 0.4 Units of RQ1 RNase-free DNase (Promega, Madison, WI, USA). A 100 μl reaction volume was used, which contained final concentrations of 50 mM Tris (pH 7.5), 10 mM $MnCl_2$, and 50 μg/ml bovine serum albumin (BSA). The mixture was incubated for 30 min at 37 °C. After purification by using phenol/chloroform extraction and ethanol precipitation, the fragments were blunted by using a DNA blunting kit (Takara Shuzo, Shiga, Japan) according to the manufacturer's instructions. The fragments were then electrophoresed in an 18% polyacrylamide gel, and the part of the gel that contained DNA that was 18–30 bp in length was excised, sliced into small pieces, soaked with TE and shaken overnight at room temperature. After separating the eluate from the gel by using an UltraFree-MC sterile centrifuge filter unit with a 0.22 μm GV Durapore column (Millipore, Billerica, MA, USA), the fragments were purified by filtrating the eluate with a NAP10 column (Amersham Bioscience, Piscataway, NJ, USA), lyophilized, and resolved in 20 μl TE. One microliter of the solution was ligated with linker I and linker II by using a ligation kit (Takara Shuzo) as described by the manufacturer (linker I was obtained by annealing 5′-gtg gaa agg acg ttt aaa aa-3′ and 5′-ttt tta aac gtc ctt t-3′; linker II was obtained by annealing 5′-ttt tta agg ctt ttc tcc-3′ and 5′-cct tgg aga aaa gcc tta aaa a-3′), and purified by using a G25 column (Amersham Bioscience). The ligated fragments were further amplified by using 5′-ctt tat ata tct tgt gga aag gac g-3′ and 5′-cta taa ata tcc ctt gga gaa aag cc-3′.

Preparation of the parallel-type siRNA expression vectors

The parallel-type siRNA expression vector inserts contained a human U6 promoter, an siRNA expression sequence prepared as described above, and a mouse U6 promoter. To obtain these inserts, first we amplified the human U6 promoter and mouse U6 promoter. Primers used for amplification were 5′-ggg aat tca cct gcc ggc cac cgt ttt ccc agt cac gac gtt g-3′ and 5′-aga tat ata aag cca aga aat cga aat a-3′ (human U6 promoter), and 5′-gga tat tta tag tct caa aac aca caa tta-3′ and 5′-ggc gga tcc acc tgc cgg cca ccg agc gga taa caa ttt cac aca gg-3′ (mouse U6 promoter). Then the human U6 promoter, mouse U6 promoter and the siRNA expression sequence were assembled together by using 5′-ggg *aat tca* cct gcc ggc cac cgt ttt ccc agt cac gac gtt g-3′ and 5′-ggc *gga tcc* acc tgc cgg cca ccg agc gga taa caa ttt cac aca gg-3′ to obtain parallel-type siRNA expression vector inserts with *EcoRI* and *BamHI* restriction sites (shown in italics). The inserts were cut and inserted into the *EcoRI* and *BamHI* sites of a pcPUR-U6i cassette constructed previously in our laboratory, and the plasmids were transformed into *Escherichia coli* cells.

For negative controls, we used a parallel-type siRNA expression vector expressing a DsRed sequence that does not have any suppressive effect and a parallel-type siRNA expression vector against *Renilla* luciferase. The sense sequence of the siRNA was 5′-acc cgc gac ggc gtg ctg aag ggc gt-3′ for the inactive DsRed siRNA, and 5′-agc gcg gtg tat tat acc t-3′ for the siRNA against *Renilla* luciferase.

Transfection and quantification of DsRed and GFP activities

To screen for siRNA target sequences with high RNAi activities against DsRed and GFP, we cotransfected HeLa S3 cells cultured in 24-well tissue culture plates with 250 ng of the parallel-type siRNA expression vector, or the vector groups containing approximately five *E. coli* colonies per group, and 100 ng pDsRed2-c1 (Clontech)

and 25 ng pHygEGFP (Clontech). Transfections were performed by using Effectene (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Two days after the transfection, cells were lysed with passive lysis buffer (Promega), and DsRed and GFP fluorescence was quantified by using Fluoroskan Ascent FL (Thermo Labsystems, Helsinki, Finland). For siRNAs against DsRed, DsRed activity was normalized against GFP activity, and *vice versa*.

Determining the siRNA target sequences

To simplify the screening process, we first screened the groups of siRNA expression vectors that contained approximately five colonies per group. We cultured colonies separately in 48-well tissue culture plates for 24 h, mixed equal amounts of the five cultures, and cultured the mixtures again overnight. The plasmids were then extracted by using Qiaprep Spin Miniprep Kit (Qiagen), and quantified for DsRed and GFP fluorescence as described above. For the groups with a more than 50% suppressive effect, we assayed each plasmid from each group separately to determine which plasmids produced high RNAi activities, and, finally, which target sequences were responsible.

Construction of short hairpin-type siRNA expression vectors based on the screening results

To construct short hairpin-type siRNA expression vectors based on the target sequences of iRed2-46**-par and iGFP98-par, we annealed sense and antisense oligonucleotides for each target sequence and inserted them into the pcPUR-U6i cassette vector. The oligonucleotide linkers used were 5′-cac cag aga gcg tca tta ctg agt tcg tgc gtg tgc tgt ccg cat gaa ctc ggt gat gac gtt ctc ttt ttt-3′ and 5′-gca taa aaa aga gaa cgt cat cac cga gtt cat gcg gac agc aca cgc acg aac tca gta atg acg ctc tct-3′ for iRed2-46**-sh, 5′-cac cgt gtt gtt gct cga taa tca tgt gtg ctg tcc atg gtt gtc ggg cag cag cac ttt tt-3′ and 5′-gca taa aaa gtg ctg ctg ccc gac aac cat gga cag cac aca tga tta tcg agc aac aac ac-3′ for iGFP98a-sh, and 5′-cac cag tag tgt gta gct tgc tga ccg tgt gct gtc cgg tcg gcg agc tgc acg ctg ctt ttt-3′ and 5′-gca taa aaa agc agc gtg cag ctc gcc gac cgg aca gca cac ggt cag caa gct aca cac tac t-3′ for iGFP98b-sh.

Preparation of total RNA from the transfected cells and Northern blotting analysis

To obtain total RNA, we first transfected HeLa S3 cells cultured in 6-well tissue culture plates with 1 μg of the siRNA expression vector. Transfection was performed by using Effectene (Qiagen), and, 2 days after the transfection, total RNA from the transfected cells was extracted by using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions.

For Northern blotting analysis, samples containing 15 μg total RNA were fractionated by polyacrylamide gel electrophoresis (PAGE; 18% polyacrylamide, 50% urea), and transferred to a Hybond-N+ membrane (Amersham Bioscience). After the membrane had dried, the RNAs were fixed by exposure to ultraviolet light. Prehybridization was performed in Northern stock buffer (30% formaldehyde, 1× Denhardt's solution, 5× SSC, 0.5% sodium dodecyl sulfate (SDS), 10% dextran sulfate) containing 200 μg/ml salmon testes DNA (Sigma Aldrich, St. Louis, MO, USA) at 36 °C for 1 h. Hybridization was performed at 36 °C for more than 3 h after the addition of the DNA probes. The probes used were 5′-aga gaa cgt cat cac cga gtt cat gc-3′ for the detection of iRed2-46-par and iRed2-46**-sh antisense, and 5′-agc agc gtg cag ctc gcc gac cct gct gcc cga caa cca t-3′ for the detection of iGFP98-par and iGFP98a-sh antisense, labeled with [^{32}P]ATP (Amersham Bioscience) by T4 polynucleotide kinase (Takara Shuzo) prior to hybridization. After hybridization, membranes were washed twice with 2× SSC containing 0.1% SDS at 36 °C and analyzed by using a Bio-Image analyzer (BAS1000; Fuji Photo Film, Tokyo, Japan).

Dual luciferase assay for confirming cytotoxicity

To confirm the cytotoxicity of the clones that produced reductions in both DsRed and GFP fluorescence, we performed another reporter assay with firefly and *Renilla* luciferase expression vectors. A 250 ng quantity of each siRNA expression vector was cotransfected with 50 ng of firefly luciferase expression vector (pGL3; Promega) and 15 ng of *Renilla* luciferase expression vector (pRL-RSV) [21]. Transfections were performed by using Effectene and after 24 h the transfection firefly and *Renilla* luciferase activities were measured by using the dual luciferase system (Promega) according to the manufacturer's instructions.

Results

Constructing siRNA expression vectors by using fragmentized target genes opens up the possibility of constructing siRNA expression libraries from cDNAs. There are two strategies that could be potentially used to build such libraries: one is to construct parallel-type siRNA expression vector libraries, and the other is to construct hairpin-type siRNA expression vector libraries. The latter strategy has been recently described by Shirane *et al.* [20] and Luo *et al.* [22]. Indeed, we have tried to construct hairpin-type siRNA expression libraries, but we found that the vectors are genetically unstable in *E. coli* cells due to the palindromic DNA structure formed between the sense and antisense coding strands of the vectors. Because the

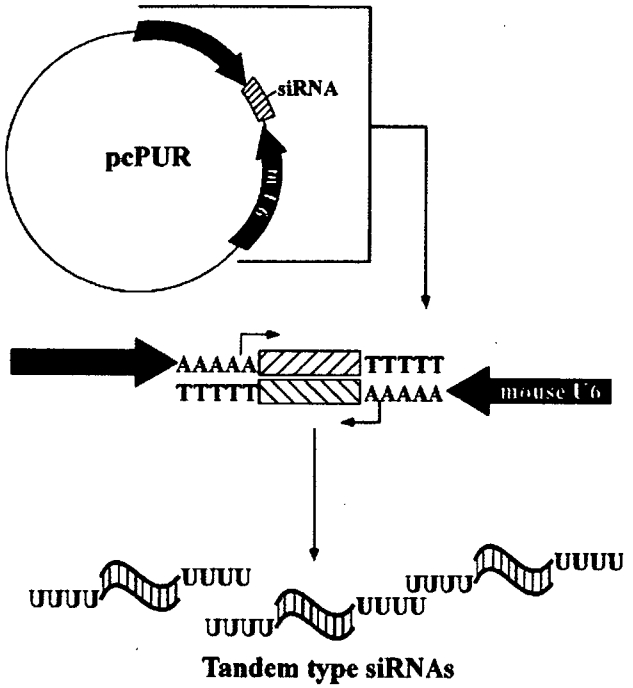

Figure 1. Parallel-type siRNA expression vector. The siRNA coding sequence is inserted between a human U6 promoter and a mouse U6 promoter. The two promoters are in opposite directions. To terminate the transcription, a stretch of Ts (transcription terminator of the Pol III promoter) is inserted. Transcription start sites are shown by the arrows. During expression, the sense strand of siRNA is expressed by one of the two promoters, and the antisense strand by the other. Expression results in tandem-type siRNAs

sense and antisense sequences match perfectly, the frequency of mutations and deletions in the dsRNA coding sequences increases [11]. Therefore, we tried another strategy, construction of parallel-type siRNA expression libraries, in which the siRNA expression sequences originate from the fragmentized target gene. A schematic diagram of the parallel-type siRNA expression vector is shown in Figure 1, and a diagram of the construction process is shown in Figure 2.

Construction of the parallel-type siRNA expression libraries

To develop a model system, we constructed parallel-type siRNA expression libraries against DsRed and GFP. Fragments of DsRed and GFP were obtained by digesting PCR products amplified from the coding sequence of each gene with DNase I. An appropriate amount of DNase and the appropriate digestion time were determined for each digestion in advance (data not shown). After ligation and two rounds of PCR, DNA fragments containing an *EcoRI* restriction site, the human U6 promoter, an 18–30-bp fragment originating from the target gene, the mouse U6 promoter and a *BamHI* restriction site were generated. Libraries were obtained after the insertion of these fragments into the *EcoRI* and *BamHI* sites of the pcPUR-U6i cassette vector.

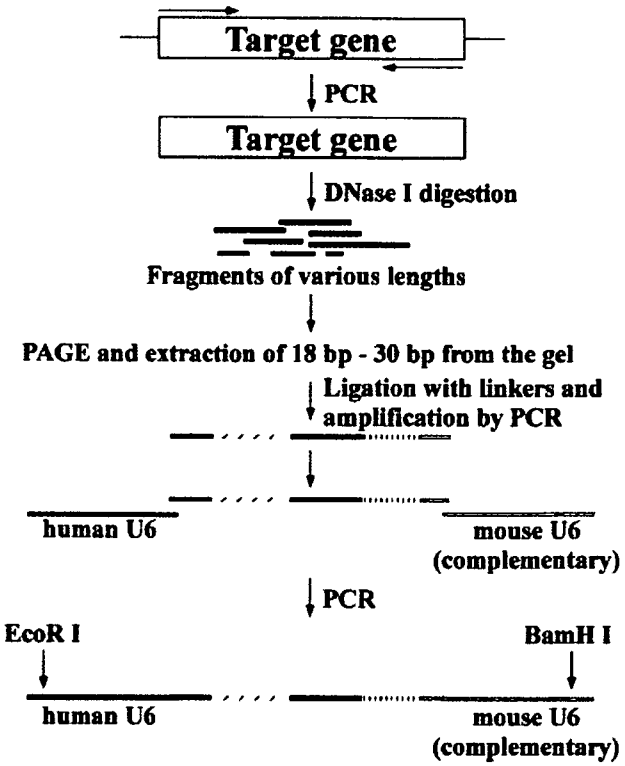

Figure 2. Strategy for constructing parallel-type siRNA expression libraries from a fragmentized target gene. The target gene is amplified by PCR, and digested by DNase I for an appropriate amount of time to generate fragments of various lengths. Fragments are fractionated by PAGE; then 18–30-bp fragments are extracted, purified and ligated with linkers. After being amplified by PCR, the ligated linkers, the human U6 promoter and the mouse U6 promoter (complementary) are assembled together to generate insert fragments containing the human U6 promoter, the fragmentized target gene and the mouse U6 promoter. Inserted fragments are cut with *EcoRI* and *BamHI*, and inserted into the backbone vector

For negative controls, we used parallel-type siRNA expression vectors expressing siRNA that did not affect the activity of either DsRed or GFP.

Screening the groups of plasmids with high RNAi activities

To simplify the screening process, we first assayed groups of five or more parallel-type siRNA expression vectors. The groups were limited to mixtures of five clones because, in an experiment with a shRNA expression vector that was known to have a high RNAi activity against DsRed, a mixture of 50 ng of the shRNA expression vector and 200 ng mock vector (i.e. a mixture of one part high RNAi activity shRNA expression vector to four parts inactive shRNA expression vector) resulted in approximately 50% suppression of DsRed activity (data not shown), whereas a mixture of 25 ng shRNA expression vector and 225 ng mock vector (one part high RNAi activity shRNA expression vector to nine parts inactive shRNA expression vector) did not produce an obvious suppression effect (only approximately 20% suppression).

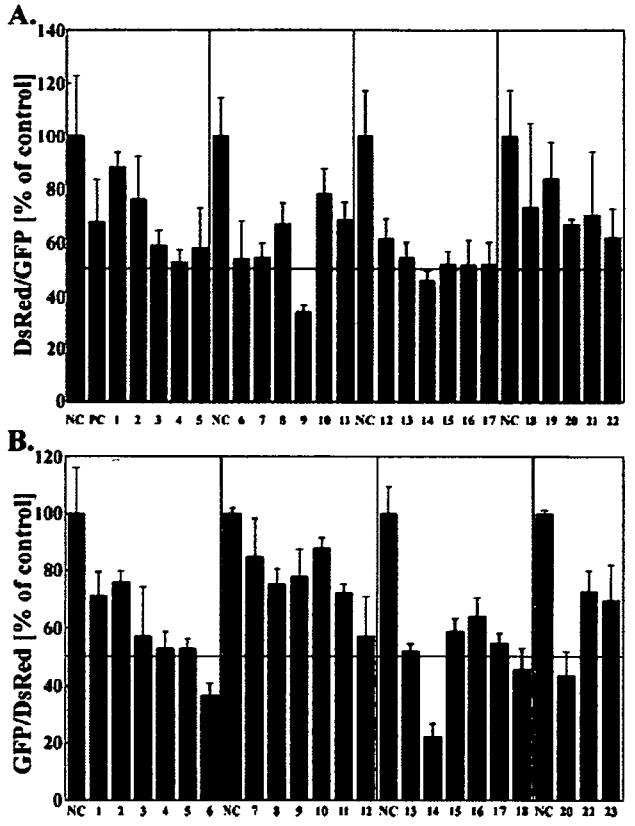

Figure 3. Results of group screenings. (A) Results of group screenings for siRNA against DsRed. Groups with more than 50% suppression of DsRed activity compared with the negative control were selected for further assay. (B) Results of group screenings for siRNA against GFP. Groups with more than 50% suppression of GFP activity compared with the negative control were selected for further assay

We cotransfected HeLa S3 cells with the vector groups (a mixture of five siRNA vectors) and the pDsRed2-c1 (a reporter plasmid for DsRed) and pHygEGFP (a reporter plasmid for GFP) vectors. Two days later, the activities of DsRed and GFP were quantified. We screened 22 vector groups (equal to more than 110 siRNA expression vectors) for DsRed and 21 groups for GFP. The results of the screening for siRNAs targeting DsRed are shown in Figure 3A, and those for GFP are shown in Figure 3B.

Determining the target sequences with high RNAi activities

To determine which plasmids from the groups contained target sequences with high RNAi activities, and, furthermore, to determine the target sequences, we selected groups that suppressed DsRed or GFP activity by more than 50%, and assayed each plasmid in the group. The limit was set at 50% because, as described earlier, a mixture of 50 ng shRNA expression vector and 200 ng mock vector suppressed the activity of DsRed to approximately 50%. One important point is that, as will be discussed later, it is possible that there would be clones that could induce cytotoxicity that might influence the expression levels of proteins. To avoid selecting groups that might contain such clones, when selecting groups for further assay we chose only those that suppressed the target gene (DsRed or GFP), but that left the activity of the other reporter unchanged (compared with the negative control). From group 9 of DsRed and group 20 of GFP, we found two plasmids, namely iRed2-46-par and iRed2-50-par, that had an approximately 80% suppressive effect against DsRed (Figure 4A), and one plasmid, namely iGFP98-par, that had an approximately 60% suppressive effect against GFP (Figure 4B).

When determining the sequences of these plasmids, we found that iRed2-46 actually came from two different colonies (we termed them iRed2-46*-par and iRed2-46**-par), and, as shown in Figure 4C, only iRed2-46**-par produced the suppressive effect. For iGFP98-par, the sequence actually came from two fragments that originated from different places in the GFP gene (namely part a and part b), but still produced a very obvious suppressive effect. Furthermore, these plasmids specifically suppressed the target genes (DsRed or GFP) without affecting the expression levels of the other. The target sequences of iRed2-46**-par, iRed2-50-par and iGFP98-par are shown in Figure 4D. The confocal microscopic images shown in Figure 4E for DsRed and Figure 4F for GFP show results that are consistent with those of the quantitative assays shown in Figures 4A and 4B.

Construction and assay of hairpin-type siRNA expression vectors

Hairpin-type siRNAs have been reported to show stronger suppressive effects than tandem-type ones with the same target sequence at low concentrations of plasmid [23]. Because our parallel-type siRNA expression vectors expressed tandem-type siRNAs, we expected that by constructing shRNA expression vectors based on the selected target sequences from the parallel-type siRNAs we could obtain even stronger RNAi effects. We constructed shRNA expression vectors based on the target sequences of iRed2-46**-par and iGFP98-par. Because the iGFP98-par target sequences came from two different GFP fragments, we constructed two hairpin-type siRNA expression vectors based on each of these, namely iGFP98a-sh and iGFP98b-sh. The structures of the shRNAs expressed from these vectors are shown in Figure 5A. To prevent the formation of palindrome structures caused by perfect matching between the sense and antisense strands, we designed the vectors with four to five C to T mutations and/or A to G mutations as described previously [10,11,23].

To determine the efficacy of inducing RNAi by using the shRNA expression vectors, we cotransfected HeLa S3 cells with the shRNA expression vectors together with the GFP and DsRed reporter vectors and assayed the GFP and DsRed activities 2 days later. For siRNA expression vectors against DsRed, the activities of DsRed

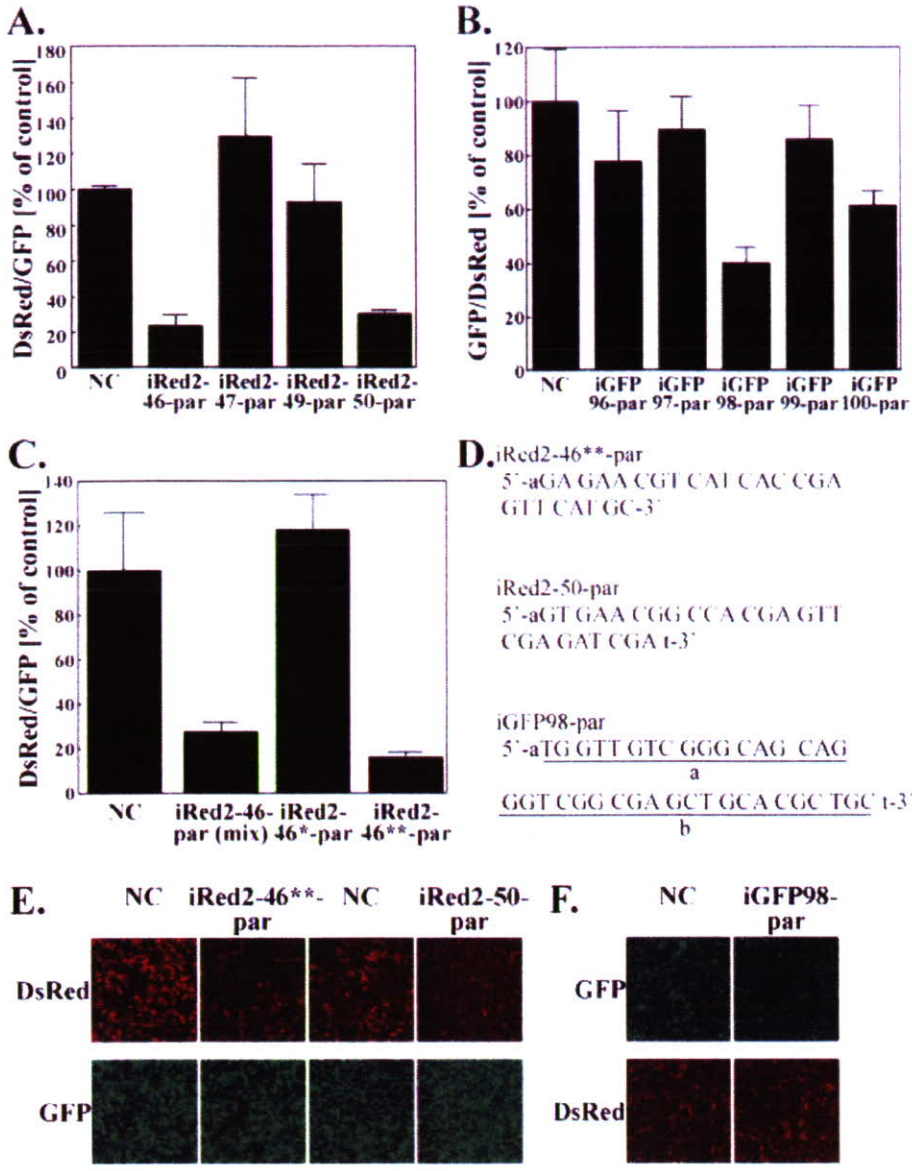

Figure 4. Identifying clones with high RNAi activities. (A) Further assay of each siRNA expression vector against DsRed from group 9: lane 1, negative control; lane 2, iRed2-46-par; lane 3, iRed2-47-par; lane 4, iRed2-49-par; lane 5, iRed2-50-par. iRed2-46-par and iRed2-50-par produced a nearly 80% suppression effect compared with the negative control, whereas others did not produce any RNAi effect. (B) Further assay of each siRNA expression vector against GFP from group 20: lane 1, negative control; lane 2, iGFP96-par; lane 3, iGFP-97-par; lane 4, iGFP98-par; lane 5, iGFP99-par; lane 6, iGFP100-par. Compared with the negative control, iGFP98-par produced a 60% suppression effect, whereas others did not produce any obvious suppression. (C) Determination of which clone of iRed2-46-par produced the suppressive effect: lane 1, negative control; lane 2, iRed2-46-par (mix); lane 3, iRed2-46*-par; lane 4, iRed2-46-par. Because iRed2-46-par was a mixture of two clones, we assayed each clone and determined that iRed2-46**-par was the one that produced the suppression effect (nearly 80%). (D) Sequences of iRed2-46**-par, iRed2-50-par and iGFP98-par. Sequences shown in capital letters are fragments originating from the target DNAs. (E) Confocal microscopic images of siRNA expression vector against DsRed: lane 1, negative control: lane 2, iRed2-46**-par; lane 3, negative control; lane4, iRed2-50-par (F) Confocal microscopic images of siRNA expression vector against GFP: lane 1, negative control; lane 2, iGFP98-par. For both DsRed and GFP, observations from the confocal microscopic images were consistent with the assay results**

were normalized against GFP expression (Figure 5B), and *vice versa* (Figure 5C). As shown in Figure 5B, compared with the negative control (lane 1), the hairpin-type siRNA, iRed2-46**-sh (lane 3), had an obviously higher suppressive effect (approximately 90%), whereas the parallel-type siRNA, iRed2-46**-par (lane 2), produced an approximately 80% suppressive effect. The same results were obtained with shRNA expression vectors against GFP (Figure 5C): compared with the negative control (lane 1), iGFP98-par (lane 2) produced approximately 60% suppression of GFP activity, whereas one of the hairpin-type expression vectors (iGFP98a-sh, lane 3) produced a suppression effect of nearly 80%, and the other one (iGFP98b-sh) did not produce a significant suppressive effect (about 40%, data not shown). The confocal microscopic images of the hairpin-type siRNA expression vectors are shown in Figures 5D (DsRed) and 5E (GFP).

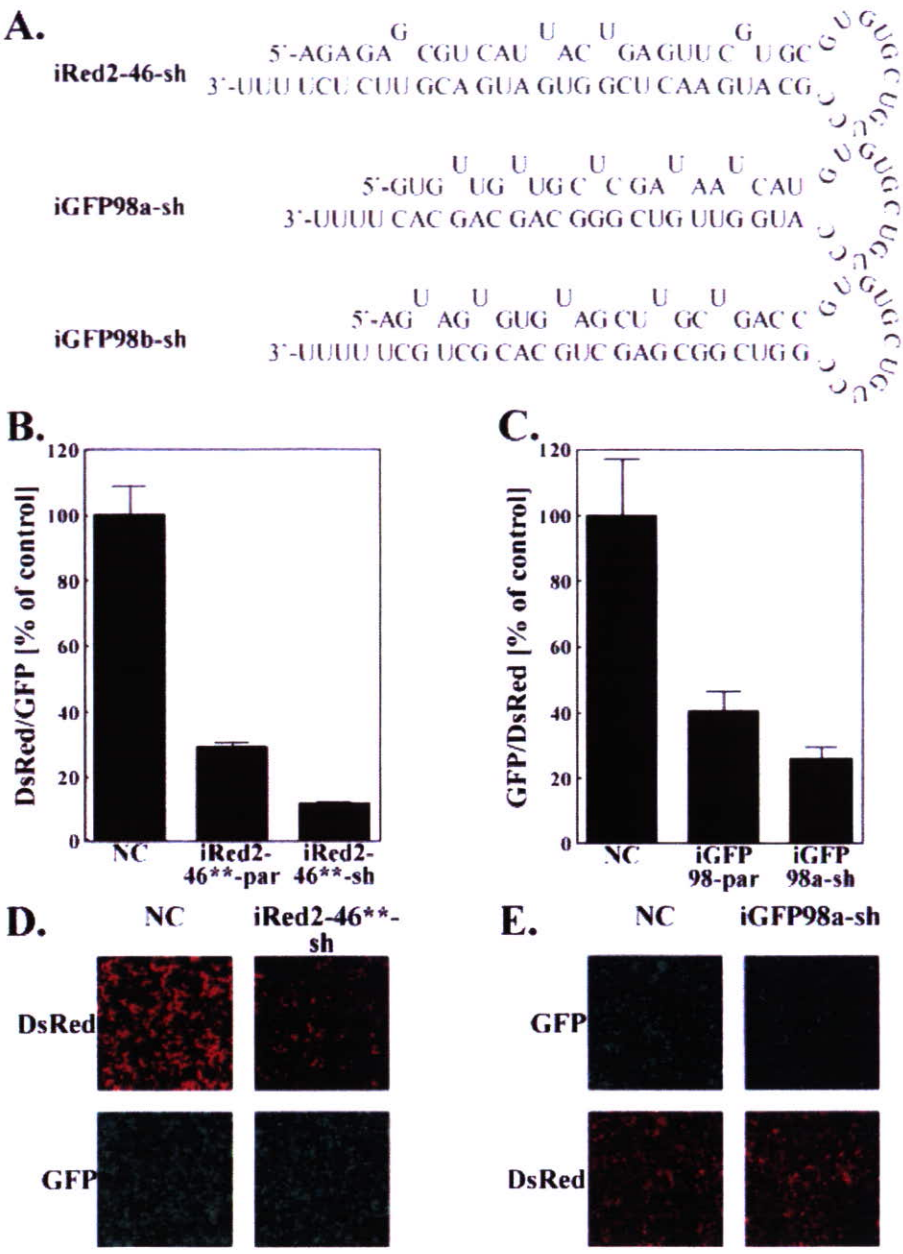

Figure 5. Comparison between parallel- and hairpin-type siRNA expression vectors. (A) Structures of hairpin-type siRNA expressed from the vectors constructed based on the target sequences from screening results: iRed2-46**-sh, iGFP98a-sh and iGFP98b-sh. Four to five A to G mutations and/or C to U mutations were inserted into the sense strand to prevent the formation of a palindromic DNA structure between the sense and antisense strands. (B) Comparison of RNAi effects for parallel- and hairpin-type siRNA expression vectors against DsRed: lane 1, negative control; lane 2, iRed2-46**-par; lane 3, iRed2-46**-sh. Compared with the negative control, iRed2-46**-par produced a nearly 80% suppression, whereas iRed2-46**-sh produced an approximately 90% suppression. (C) Comparison of RNAi effects for parallel- and hairpin-type siRNA expression vectors against GFP: lane 1, negative control; lane 2, iGFP98-par; lane 3, iGFP98a-sh. Compared with the negative control, iGFP98-par produced approximately 60% suppression, whereas iGFP98a-sh produced nearly 80% suppression. (D) Confocal microscopic images of hairpin-type siRNA expression vector against DsRed: lane 1, negative control; lane 2: iRed2-46**-sh. (E) Confocal microscopic images of hairpin-type siRNA expression vector against GFP: lane 1, negative control; lane 2, iGFP98a-sh

Northern blots of transcripts from parallel- and hairpin-type siRNA expression vectors

In RNAi, dsRNAs are processed into 21–23-bp siRNAs by Dicer [6,7]. The processed siRNAs are incorporated into RISC. The RISC that possesses one strand of siRNA recognizes and cleaves the target mRNA in a sequence-specific manner. To examine the processing of RNAs expressed from our parallel- and hairpin-type siRNA expression vectors, we subjected the siRNAs expressed to Northern blotting. We used probes with sense sequences and thus detected the antisense strands. The results are shown in Figures 6A (for siRNAs against DsRed) and 6B (for siRNAs against GFP). For both target genes, we could not detect any band in the negative control (lane 1 of

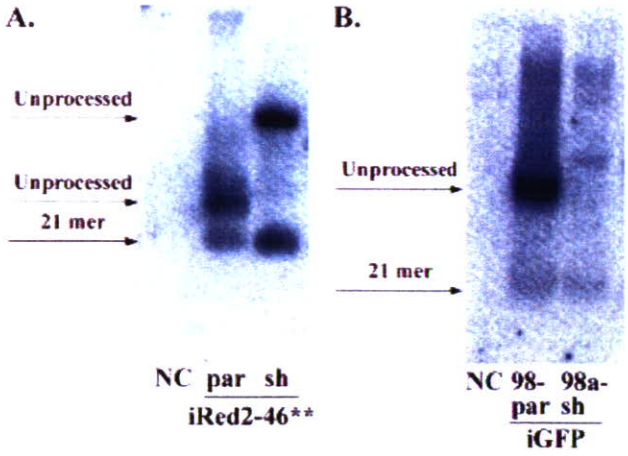

Figure 6. Northern blotting results of parallel- and hairpin-type siRNA expression vectors. (A) Northern blotting results for siRNA expression vectors against DsRed: lane 1, negative control; lane 2, iRed2-46**-par; lane 3, iRed2-46**-sh. The probe used has the same sequence as the sense strand; thus, the RNA fragments detected there were the antisense strands. 21-mer fragments could be detected in both lanes 2 and 3, indicating that both tandem-type and hairpin-type siRNAs were processed into 21-mers. (B) Northern blotting results of siRNA expression vectors against GFP: lane 1, negative control; lane 2, iGFP98-par; lane 3, iGFP98a-sh. The probe used has the same sequence as the sense strand, thus, the RNA fragments detected there were the antisense strands. 21-mer fragments could be detected in both lanes 2 and 3, indicating that both tandem-type and hairpin-type siRNAs were processed into 21-mers

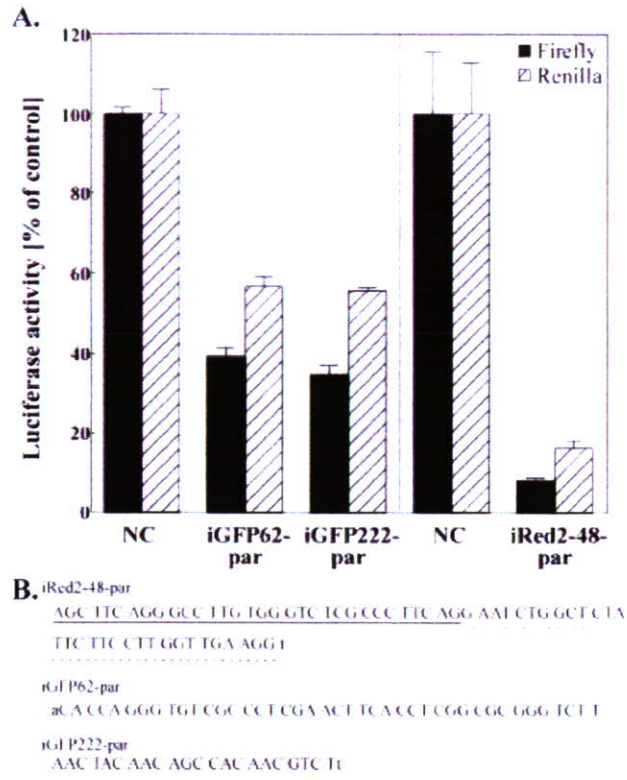

Figure 7. (A) Cytotoxicity produced by some clones. Cells were cotransfected with the clones and firefly and *Renilla* luciferase expression vectors: lane 1, negative control; lane 2, iRed2-48-par; lane 3: negative control; lane 4: iGFP62-par; lane 5: iGFP222-par. Compared with the control, iRed2-48-par, iGFP62-par, and iGFP222-par, which did not express siRNAs against either firefly or *Renilla* luciferase, produced obvious reductions in both values, indicating that cytotoxicity and/or nonspecific protein suppression occurred. (B) Sequences of clones producing cytotoxicity: iRed2-48-par, iGFP62-par, and iGFP222-par. Sequences shown in capital letters are fragments originating from the target DNA. For iRed2-48-par, the sequence underlined with a solid line originated from the DsRed sequence, and the part underlined with a dotted line originated from the tBid sequence

both figures), indicating that our probes did not show any nonspecific hybridization. Lanes 2 (parallel-type) and 3 (hairpin-type) in both figures show processed strands of the same length (21 bp), indicating that the RNAs were processed into 21-bp siRNAs regardless of the original structure.

Some clones that express different target sequences and lengths of siRNAs show cytotoxicity

Additionally, we found that some clones obtained from the library screening suppressed both DsRed and GFP activity, regardless of their origin. To confirm that cytotoxicity was occurring, we cotransfected cells with these clones and firefly luciferase and *Renilla* luciferase reporter plasmids, which do not have any relationship with the origin of the siRNA target sequences, and performed a dual luciferase assay. As shown in Figure 7A, these clones also produced suppressive activities with respect to both firefly and *Renilla* luciferase, indicating that they inhibit gene expression nonspecifically. The lengths of siRNAs expressed from these clones varied from 23 to 64 bp (Figure 7B), indicating that the cytotoxicity produced by these clones might be caused not only by their length, but also by the sequences expressed, as reported recently elsewhere [24,25].

In summary, we showed here that our system can be used to determine preferable RNAi target sequences regardless of the original sequences of the target genes. Furthermore, by utilizing this system, we also found clones with cytotoxic effects, which varied with respect to both the length and the sequences of the target sequences.

Discussion

In the present study, we have described for the first time a screening process for preferable siRNA target sequences by using a parallel-type siRNA expression vector system with the target sequences originating from the fragmentized target gene. We have demonstrated here that this system could be used to determine highly effective siRNA target sequences, regardless of the sequences of the target gene.

We chose to construct siRNA expression vector libraries using fragmentized target genes for a number of reasons. First, by using this system, all possible target sequences

can be screened, enabling us to determine the strongest target sequence for a specific target gene. Although algorithms can now be used to predict strong target sequences [26], we believe that using an experimental approach that can cover all of the possible target sequences of various lengths is also important, especially for therapeutic applications, in which the strongest target sequence might be the best choice. Moreover, for targeting virus RNAs, selection of the best sequences using algorithms remains difficult, most probably because of tight RNA structures especially at the conserved regulatory regions. In the case of viral RNAs, using our strategy it would be possible to find effective target sequences experimentally. Furthermore, this system allows the construction of siRNA expression vector libraries, and finally determines the target sequences, without knowledge of the whole target gene sequence in advance. By using this system, we were able to simultaneously determine both the preferable target sequences and the target sequences that could induce cytotoxicity and/or nonspecific gene suppression, and therefore should be avoided. This approach may, however, require the screening of many colonies in order to find target sequences with high RNAi activities. In the present study, we screened 110 clones of siRNA expression vectors against DsRed (716 bp), and 105 clones against GFP (720 bp), and found clones (two clones for DsRed and one clone for GFP) with high RNAi activities. We believe that by screening more clones, we could identify more target sequences with high RNAi activities.

We found that for the construction of siRNA expression vector libraries with siRNA expression fragments originating from the fragmentized target gene, the parallel type is more suitable than the hairpin type. The reasons are, first, because the parallel-type siRNA expression vector contains two promoters in opposite directions, only one siRNA expression fragment is needed to express both sense and antisense strands, while for the hairpin-type expression vector, we would need to construct a vector that can express sense strand, loop and antisense strand. In other words, by using the fragmentized target gene as a source of siRNA sequences, the hairpin-type siRNA expression vectors thereby constructed contain perfect matching sense and antisense sequences that can form palindromic DNA structures. In fact, we have also tried to construct hairpin-type siRNA expression libraries, but, as expected, the constructs were genetically unstable in *E. coli* cells due to the palindromic DNA structures. Moreover, compared with the method of constructing hairpin-type siRNA expression libraries, our system is simpler [11]. Thus, we conclude that for the purposes of identifying target sequences with high RNAi activities, a parallel-type siRNA expression library is more suitable than a hairpin-type one. Furthermore, to obtain siRNAs with higher suppressive activities, we could simply construct hairpin-type siRNA expression vectors based on the target sequences obtained from the screening. It is noteworthy that longer siRNAs (25–27 nt in length) have been recently reported to produce a greater suppressive effect than 21-nt siRNAs in some cases [27]. In our strategy, by selecting fragmentized DNAs within a range of lengths, we could construct and screen expression vectors that express siRNAs of different lengths. Thus, our strategy could be used to predict the target sequences of longer siRNAs. This could not be achieved by using a target sequence prediction algorithm, because in the algorithm, the length of the siRNA is pre-set.

Interestingly, we also found clones with nonspecific effects on the expression levels of proteins. One of the clones, iRed2-48par, caused not only decreases in protein expression level, but also cell death. Because this clone expresses a relatively long dsRNA (64 bp), we thought that it might induce an interferon response, and thus cause general protein suppression and cell death. The lengths of the dsRNAs expressed by other clones that induced cytotoxicity were 23 and 43 bp, so we hypothesize, especially for the clone expressing the 23 bp sequence, that cytotoxic mechanisms other than an interferon response might be responsible for the nonspecific effect. Some groups recently reported that dsRNAs with a particular sequence induce cytotoxicity, and that this cytotoxicity could be dependent on or independent of interferon response, regardless of the length of the dsRNAs [24,25]. Our findings are consistent with these reports.

In conclusion, we have developed an experimental approach for simultaneously identifying siRNA target sequences with high RNAi activities, and target sequences that should be avoided due to cytotoxicity. We successfully determined target sequences for two genes in a model system using DsRed and GFP reporter genes, and constructed two hairpin-type siRNA expression vectors with high RNAi activities based on those target sequences. In short, we believe that our method is a useful and simple alternative for determining siRNA target sequences.

Acknowledgements

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δEF1 Mediates TGF-β Signaling in Vascular Smooth Muscle Cell Differentiation

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Summary

Alteration in the differentiated state of smooth muscle cells (SMCs) is known to be integral to vascular development and the pathogenesis of vascular disease. However, it is still largely unknown how environmental cues translate into transcriptional control of SMC genes. We found that δEF1 is upregulated during SMC differentiation and selectively transactivates the promoters of SMC differentiation marker genes, *SM α-actin and SM myosin heavy chain (SM-MHC)*. δEF1 physically interacts with SRF and Smad3, resulting in a synergistic activation of *SM α-actin* promoter. Chromatin immunoprecipitation assays and knockdown experiments showed that δEF1 is involved in the control of the SMC differentiation programs induced by TGF-β signaling. Overexpression of δEF1 inhibited neointima formation and promoted SMC differentiation, whereas heterozygous *δEF1* knockout mice exhibited exaggerated neointima formation. It thus appears δEF1 mediates SMC differentiation via interaction with SRF and Smad3 during development and in vascular disease.

Introduction

Unlike striated muscle cells, smooth muscle cells (SMCs) are not terminally differentiated, even in adult blood vessels, and the plasticity they retain enables them to modify their phenotype in response to environmental cues (Owens et al., 2004). It is well documented that phenotypically modulated SMCs are centrally involved in the pathogenesis of vascular disease. Consequently, elucidation of the mechanisms that control the differentiated state of SMCs is critically important for understanding not only vascular development but also the pathology of vascular disease.

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A number of transcription factors have been implicated in SMC differentiation, but unlike in skeletal muscle, no single transcription factor identified so far can, by itself, induce SMC differentiation. Recent studies have clearly demonstrated that SMC gene expression is highly context dependent and is controlled not by a single SMC-specific transcription factor but by the interactions between multiple factors and cofactors, which likely form networks that selectively and coordinately control gene expression in response to environmental cues (Owens et al., 2004; Manabe and Nagai, 2003). Transcription factors and cofactors reportedly involved in such networks include SRF, GATA6, and myocardin (Miano, 2003; Owens et al., 2004); though it is still largely unknown how these factors interact within the networks to execute SMC-specific gene programs.

SMC-specific transcriptional regulatory modules very often contain the CArG element, a target element of serum response factor (SRF); indeed, in vivo expression of virtually all of the SMC-specific genes studied so far depends on CArG elements (Miano, 2003). However, SRF is ubiquitously expressed in a variety of cell-types and is necessary for expression of a large number of non-muscle genes. Thus, one critical question in the field has been: How do ubiquitously expressed factors such as SRF regulate SMC-specific transcription? One clue may come from the recent report that a transcription cofactor, myocardin, is necessary for SMC-specific SRF-dependent transcription (Wang et al., 2003). Still, it remains unclear how environmental cues translate into alterations in gene transcription that depend on SRF and myocardin.

One external factor thought to be important for control of SMC differentiation is TGF-β, which is able to induce neural crest stem cells to express SMC differentiation marker genes, such as *SM α-actin*, and to augment expression of SMC markers in cultured SMCs (Shah et al., 1996). In addition to these in vitro studies, gene knockout studies in which *TGF-β* was targeted confirmed that TGF-β signaling is essential for proper vessel formation (Dickson et al., 1995), while targeting *endoglin*, *ALK-1*, *ALK-5* (TGF-β receptors), or *Smad5* resulted in reduced SMC/pericyte recruitment and proliferation within vessels (Li et al., 1999; Oshima et al., 1996; Yang et al., 1999). TGF-β has also been shown to be crucially involved in SMC differentiation and proliferation in vascular injury models (Mallat et al., 2001). And given the importance of SRF in SMC-specific transcriptional programs, it seems likely that it, too, is involved in TGF-β-dependent transcriptional control. Indeed, one earlier report showed that two CArG boxes in the *SM α-actin* promoter were necessary for TGF-β responsiveness (Hautmann et al., 1997). The molecular basis for the effects of TGF-β on SRF-dependent SMC gene expression is not yet clear, however.

δEF1 (also called ZEB-1) is a transcription factor that contains two clusters of zinc fingers and a homeodomain and was first identified as a factor binding to an enhancer in the chicken *δ1-crystallin* gene (Funahashi et al., 1993; Postigo and Dean, 1997). In mouse embryo,

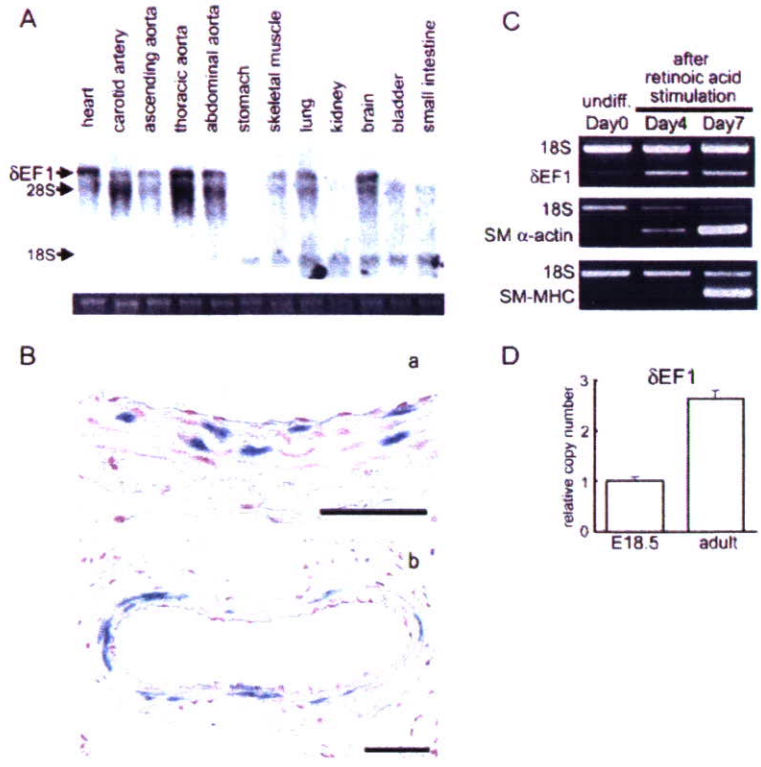

Figure 1. δEF1 Expression in Tissues and during SMC Differentiation

(A) Northern blot analysis of tissue distribution of δEF1 mRNA in adult rats.

(B) δEF1 expression in the cardiovascular system. *δEF1* knockout mice harbor a *LacZ* reporter gene controlled by the δEF1 regulatory program (Takagi et al., 1998). Tissues taken from adult $\delta EF1^{+/-}$ mice were subjected to X-gal staining and counterstained with nuclear fast red. Cross-sections of the thoracic aorta (Ba) and femoral artery (Bb) are shown. Scale bars, 50 μm.

(C) δEF1 expression during SMC differentiation of A404 cells. Expression of δEF1, SM α-actin, and SM-MHC was analyzed by semiquantitative multiplex PCR with primer sets for the gene of interest and an internal control (18S rRNA).

(D) Real-time PCR analysis of δEF1 expression in the aortas of embryos (E18.5) and adult mice. The relative numbers of δEF1 transcripts were normalized to those of 18S. Bars indicate relative copy number and SE.

δEF1 expression was first detected in the headfold and the presomitic and lateral plate mesoderm on embryonic day (E) 8.5 and in the derivatives of the cranial neural crest and limb buds on E9.5 (Takagi et al., 1998). Given that vascular SMCs are derived from a variety of embryonic progenitors, including lateral mesoderm, cranial mesenchyme, and the neural crest (Majesky, 2003), the expression pattern of δEF1 is suggestive of a role in SMC biology, though the function of δEF1 in the cardiovascular system has not yet been explored. Interestingly, recent studies have shown that δEF1 interacts with Smad proteins, the basic components of intracellular TGF-β signaling pathways.

In the present study, we identified *δEF1* as a candidate gene that might control SMC differentiation, and subsequent analyses showed that δEF1 is selectively expressed in vascular SMCs and that it controls SMC-specific gene expression by mediating TGF-β signaling and SRF-dependent transcription. Overexpression of δEF1 inhibited neointima formation and promoted SMC differentiation, whereas heterozygous *δEF1* knockout mice exhibited exaggerated neointima formation. Taken together, these findings suggest that δEF1 plays an important role in the control of SMC differentiation occurring in response to environmental cues during development and in vascular diseases.

Results

δEF1 Is Preferentially Expressed in Vascular SMCs and Is Developmentally Regulated

With the aim of identifying transcription factors involved in SMC differentiation, we searched databases for transcription factors containing C_2H_2-type zinc fingers and discovered several that might be expressed in SMCs. Among these was δEF1, which in adult rats is expressed in the aorta, heart, carotid artery, brain, and skeletal muscle but not in visceral smooth muscle tissues, such as the bladder, stomach, and small intestine (Figure 1A). Because this finding suggested that δEF1 might be expressed in vascular SMCs, we next carried out a more detailed analysis of the localization of δEF1 in cardiovascular tissues. Tissues obtained from *δEF1* knockout mice harboring a *LacZ* reporter gene whose expression mimicked endogenous *δEF1* expression (Takagi et al., 1998) were stained with X-gal. As expected, positive staining was observed in SMCs located in the media of arteries (Figure 1B) as well as in cardiomyocytes (data not shown). Positively stained cells were also occasionally observed in veins (data not shown). Arterial endothelial cells were not positively stained (Figure 1B).

To analyze δEF1 expression during SMC differentiation, we utilized the A404 cell line, which is an embryonic carcinoma P19-derived in vitro SMC differentiation system (Manabe and Owens, 2001b). Undifferentiated A404 cells do not express SMC differentiation marker genes (Figure 1C), but when stimulated with retinoic acid, they rapidly acquire SMC phenotypes and express such SMC markers as SM myosin heavy chain (SM-MHC). Likewise, expression of δEF1 was upregulated during SMC differentiation in this system (Figure 1C). In the aorta, moreover, greater expression of δEF1 was seen in adult mice than in E18.5 embryos (Figure 1D). Taken together, these findings suggest that δEF1 expression is restricted to SMCs in the arterial wall, that its expression is regulated by the differentiation state of those cells, and that, perhaps, δEF1 controls the differentiation state of SMCs.

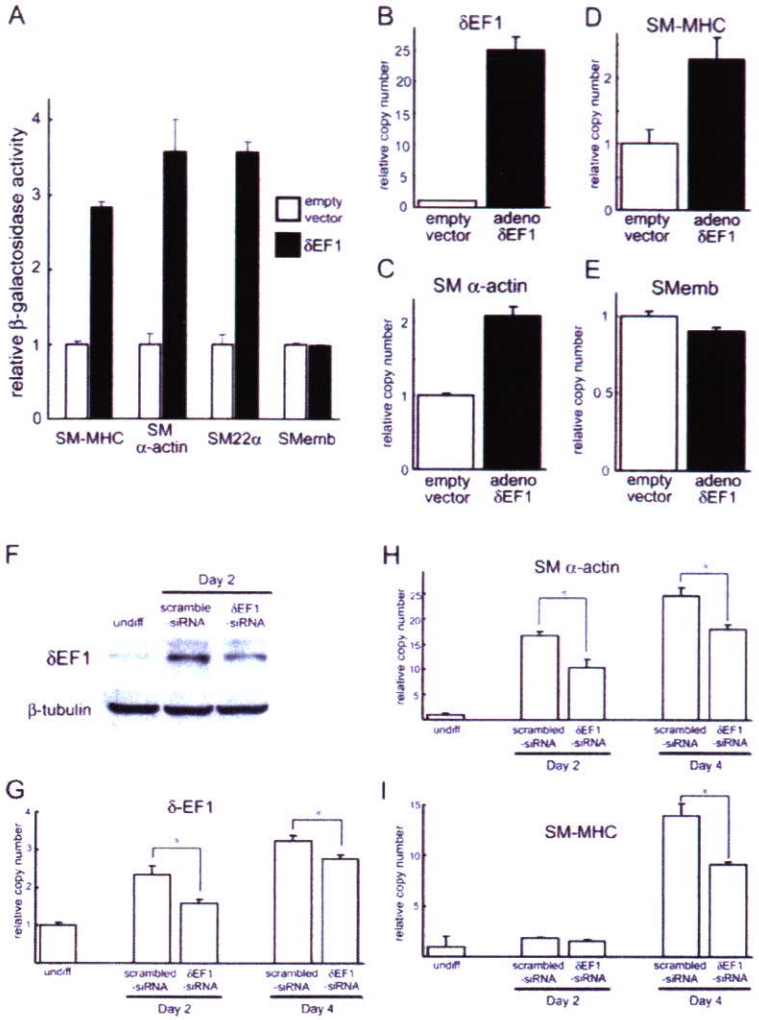

Figure 2. δEF1 Transactivated SMC Differentiation Marker Gene Promoters

(A) Effects of δEF1 on the regulatory region of SMC differentiation marker genes. Reporter genes driven by the regulatory region of indicated genes were transiently cotransfected with the δEF1 expression vector or an empty vector. The β-galactosidase activity of each reporter construct cotransfected with δEF1 expression vector was normalized to that cotransfected with the empty vector. Bars indicate relative β-galactosidase activity and SE.

(B–E) Effect of δEF1 on expression of endogenous SMC differentiation marker genes. Cultured SMCs were infected with either the δEF1 adenovirus or empty adenovirus at 20 MOI and then harvested 36 hr after infection. mRNA expression was analyzed by real-time RT-PCR. δEF1 detected in (B) included transcripts from both endogenous and exogenous genes. The copy number of each transcript was normalized to that of 18S, after which the expression was further normalized to that of cells infected with empty adenoviral vector. Bars indicate relative copy number and SE.

(F–I) Effects of δEF1 knockdown on SMC differentiation of A404 cells. A404 cells were transfected with either δEF1-siRNA or scrambled-siRNA. Twenty-four hours after transfection, the cells were treated with all-*trans* retinoic acid. Two and 4 days after the initiation of differentiation, the cells were harvested and subjected to Western analysis of δEF1 (F) and real-time PCR analysis of δEF1 (G), SM α-actin (H), and SM-MHC (I). Bars indicate relative copy number and SE.

δEF1 Controls SMC Differentiation Marker Gene Expression

To test the hypothesis that δEF1 controls the differentiated state of SMCs, we used transient transfection analysis to examine its contribution to the transcriptional regulation of SMC differentiation marker gene expression (Figure 2A). We found that δEF1 transactivated the transcriptional regulatory regions of *SM-MHC* (−4.2 to +11.6 kb) and *SM α-actin* (−2.6 to +2.7 kb), as well as the *SM22α* promoter (−441 to +41 bp), all of which have been shown to drive SMC-specific gene expression in vivo (Owens et al., 2004). By contrast, δEF1 did not activate the regulatory region (−5 to +7 kb) of *SMemb*, a marker gene expressed in embryonic and phenotypically modulated SMCs. Consistent with these effects on the promoter reporters, overexpression of δEF1 in cultured rat aortic SMCs with an adenoviral expression vector led to significant increases in the endogenous expression of *SM α-actin* (2.1-fold increase) and *SM-MHC* (2.3-fold), as compared to those seen in SMCs infected with empty adenovirus (Figures 2C and 2D). The expression level of SMemb was somewhat reduced in the δEF1-overexpressing cells (Figure 2E).

To further test the involvement of δEF1 in SMC differentiation, δEF1 expression was knocked down in A404 cells with siRNA, after which the cells were induced to differentiate. Due to the low transfection efficiency of A404 cells and the long durations of culture after the siRNA transfection, the levels of knockdown of δEF1 expression were modest (Figures 2F and 2G). Nevertheless, expression of SM α-actin and SM-MHC mRNA was significantly inhibited (Figures 2H and 2I), suggesting that knockdown of δEF1 delayed differentiation of A404 cells.

δEF1 Controls the *SM α-actin* Proximal Promoter

To define how δEF1 controls SMC differentiation marker gene transcription, a set of reporter plasmids encoding various *SM α-actin* and *SM-MHC* deletion constructs were cotransfected with the δEF1 expression plasmid. The results obtained with the *SM-MHC* reporter indicated that δEF1 affects the transcriptional activity of the *SM-MHC* regulatory region (−4.2 to +11.6 kb) by acting via multiple subregions within it (data not shown). On the other hand, the initial deletion analysis of the *SM α-actin* transcriptional regulatory region (−2.6 to +2.7 kb) suggested that δEF1 mainly controlled transcription of this gene via its proximal promoter (Figure 3B). To facilitate our analysis of δEF1 activity, therefore, we focused on the *SM α-actin* proximal promoter. Deletion

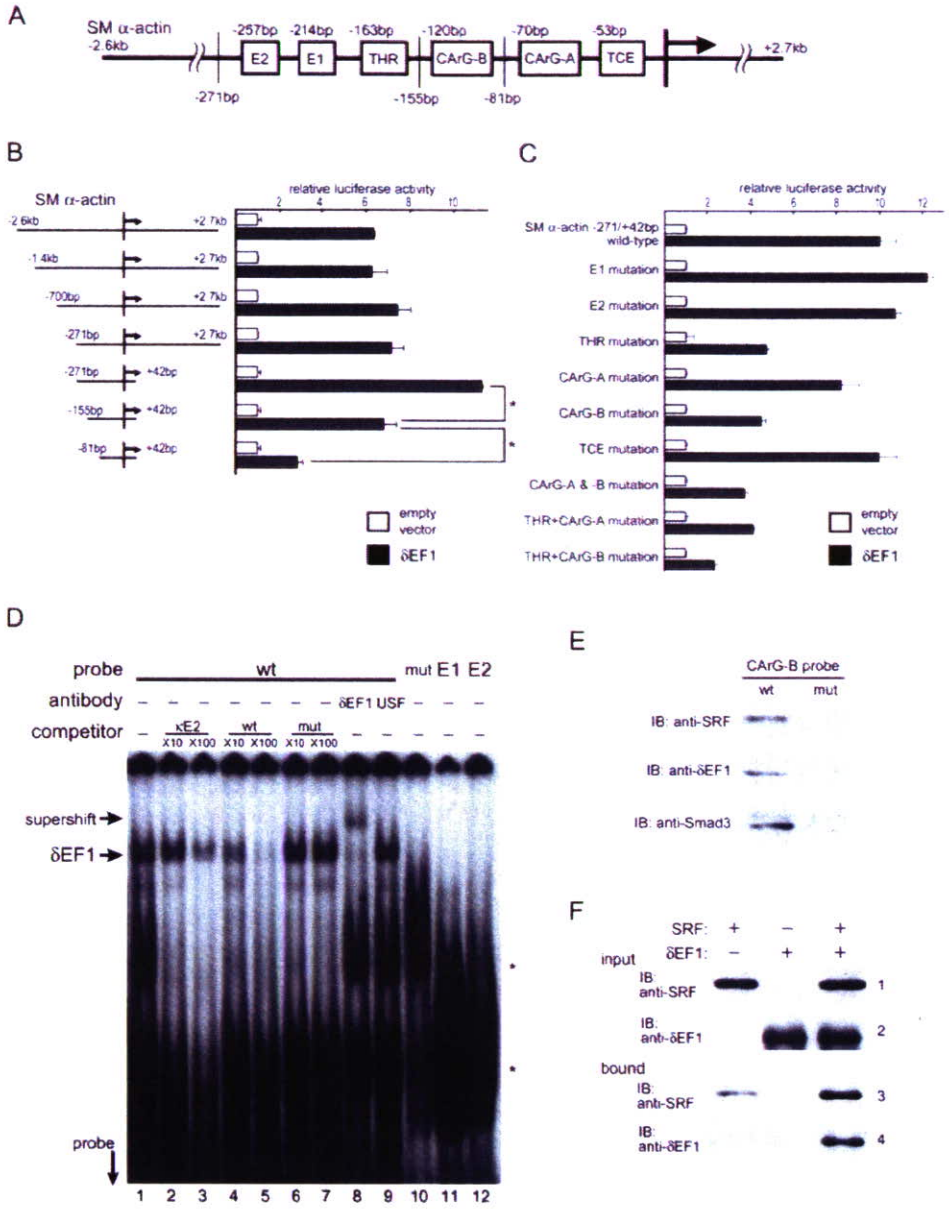

Figure 3. Analysis of δEF1-Mediated Transactivation of *SM α-actin* Promoter

(A) Schematic representation of the transcriptional regulatory regions of *SM α-actin*.

(B) Effects of δEF1 on the transcriptional activity of *SM α-actin* deletion mutants. Luciferase reporter constructs containing the indicated *SM α-actin* deletion mutants were transiently cotransfected into cultured SMCs along with either δEF1 expression vector or empty vector. Bars indicate relative luciferase activity and SE; *$p < 0.05$.

(C) Effects of mutations within the *cis*-regulatory elements on transactivation of the *SM α-actin* promoter (−271/+42) by δEF1. THR, CArGs, TCE, and E boxes were mutated within the −271/+42 bp construct. The luciferase activity of each reporter construct cotransfected with the δEF1 vector was normalized to the activity of the reporter cotransfected with empty vector. Effects of the mutations on the basal *SM α-actin* promoter activity are shown in Figure S5. Bars indicate relative luciferase activity and SE.

(D) EMSA analysis of the binding of δEF1 to the *SM α-actin* promoter. ^{32}P-labeled wild-type THR (wt), mutant THR (mut), E1, and E2 oligonucleotides were incubated with SMC nuclear extracts and subjected to EMSA. In lanes 2–7, a molar excess of the indicated cold competitor was added to the reactants; in lanes 8 and 9, anti-δEF1 or anti-USF antibody was added. Asterisks indicate nonspecific shift bands.

(E) Binding of SMC transcription factors to CArG-B. Nuclear extracts of cultured SMCs were incubated with biotinylated wild-type or mutant CArG-B probe, and the probe and bound proteins were collected with streptavidin-conjugated magnet beads. The bound proteins were subjected to SDS-PAGE and immunoblotting with anti-SRF, anti-δEF1, and anti-Smad3 antibodies.

(F) Binding of in vitro translated proteins to the CArG-B probe. SRF and/or δEF1 were incubated with the biotinylated wild-type CArG-B probe. Input proteins (1 and 2) and bound proteins (3 and 4) were immunoblotted with anti-SRF (1 and 3) or anti-δEF1 antibody (2 and 4).