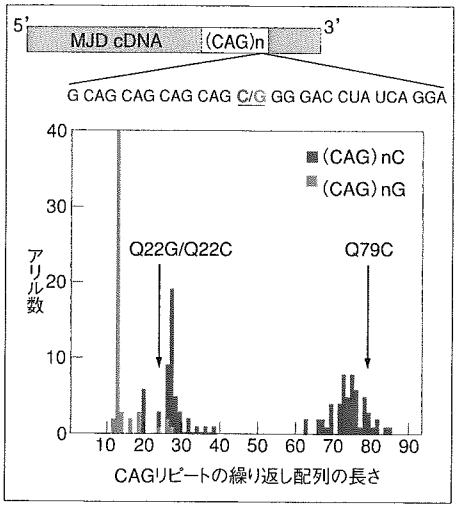
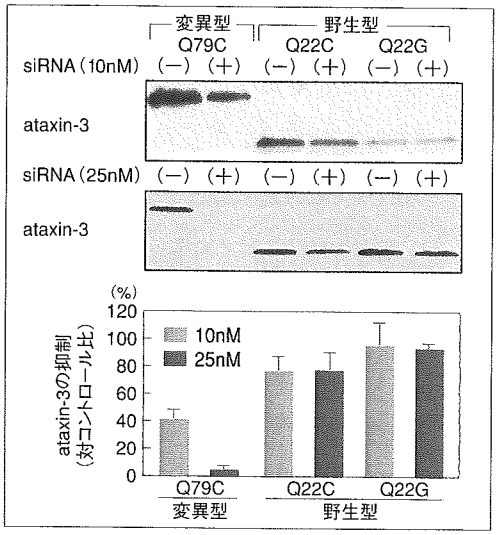
図2　Machado-Joseph 病における CAG リピートの繰り返し配列の長さと C/G の polymorphrism



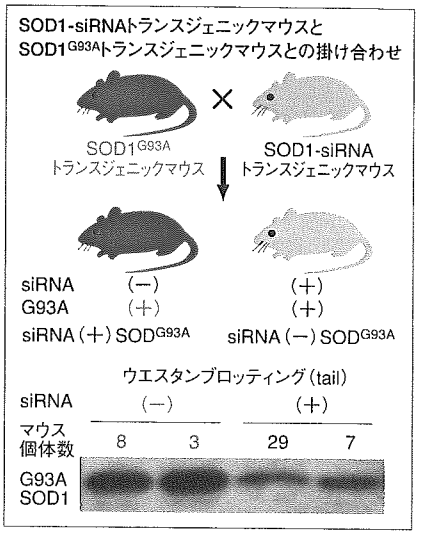
Machado-Joseph 病は MJD 遺伝子内の CAG リピートの伸長によって発症する。CAG リピートの後には G/C polymorph-ism があり、伸長した CAG リピートを持つ変異アリルはすべて G で、正常アリルでは G/C が同頻度で見られる。　（文献 2）より改変転載）

図3　MJD RNA に対する配列変異アリル特異的な1次配列非依存的な siRNA の切断



われわれのデザインした MJD siRNA はこの 1 塩基の差を認識して変異アリル（Q79C）を切断し、正常アリル（Q22G）は切断しなかった。加えて、驚いたことにこの MJD siRNA は Q79C と標的配列の全く同じのもう 1 つの正常アリル（Q22C）もわずかにしか切断しなかった。　（文献 2）より改変転載）

図4　SOD1 G93A トランスジェニックマウスの遺伝子治療



SOD1 に対する siRNA を過剰発現させたトランスジェニックマウスを ALS のモデルマウスである G93A 変異 SOD1 トランスジェニックマウスと掛け合わせることにより、変異 SOD1 蛋白の発現を 80% 以上抑制することに成功した。6 月齢の時点で ALS 症状の発症は完全に抑制されている。

のpolymorophrismがあり、これはCAGリピートの繰り返し配列の長さと関連している。長い繰り返しを持つ病的アリルはすべてCだが、短い繰り返しを持つ正常アリルでは約半数の例でGである（**図2**）。そこでわれわれはこのC/Gのpolymorphrismの標的としてsiRNAを設計して、病的アリルに特異的なsiRNAを作製した。ところが驚いたことにこのsiRNAはpolymorphrismが変異アリルと同じCである短いCAGリピートの正常アリルもあまり切断しなかった（**図3**）[2]。この機序は、CAGリピート長の変化に伴うRNAの2次構造の変化によって、siRNAの標的配列へのアクセスに差異が生じたためと考えている。

トランスジェニックマウスへの *in vivo* の有効性

SCA1のトランスジェニックマウスの小脳にsiRNA発現型アデノ随伴ウイルスを注入して、運動障害と神経変性を改善したとの報告がなされた[3]。さらに最近、SOD1に対するsiRNA発現型レンチウイルスを全身の骨格筋に注射して、逆行性に運動ニューロンにsiRNAを発現させて、G93A変異SOD1トランスジェニックマウスの発症を抑制した報告もされている[4]。われわれもSOD1に対するsiRNAを過剰発現させたトランスジェニックマウスを作製して、これをALSのモデルマウスであるG93A変異SOD1トランスジェニックマウスと掛け合わせ、全身の変異SOD1蛋白の発現を80%以上抑制することに成功した。この効果により、6月齢の時点でALS症状の発症は完全に抑制されている（**図4**）（投稿中）。

siRNA の *in vivo* へのデリバリー

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[5]。その他にも、有望なカチオニックベクターが次々に報告されている。しかし、これらの非ウイルスベクターの持続時間は短く、長期の抑制効果にはウイルスベクターが必要となる。各種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が、血液脳関門を有効に越える方法はいまだ確立していない。

siRNA の off-target 効果などの副反応

siRNAには、off-target効果、すなわち、ターゲットとした遺伝子以外に、用いた19塩基のsiRNAの配列に部分的にホモロジーのある別の遺伝子の発現を抑えてしまういわゆる交叉反応が報告されている。siRNA の配列 19 塩基中 15 塩基以上にホモロジーのある他の遺伝子において影響があったと報告された。今後この off-target効果の評価とその回避は、臨床応用する際に大きな問題になる可能性がある。

おわりに

siRNA の核酸医薬としての臨床応用の研究には、off-target effect など安全性の問題や silencing など効果の持続の問題、血液脳関門を越えるデリバリー方法など解決すべき課題はまだ多くある。しかし、siRNA の遺伝子抑制効果は顕著で、その機序は急速に解明され。基礎研究は爆発的に進んでいる。したがって、非常に近い将来に、難治性疾患での新しい治療法の開発にsiRNAの利用が突破口になることに十分に期待したい。

〔ウイルス　第55巻　第1号，pp.1-8，2005〕

総　説

1. RNAiを用いたウイルス複製抑制

横田　隆徳
東京医科歯科大学院　脳神経機能病態

RNA interference（RNAi）は2本鎖RNAiによって誘導される配列特異的な遺伝子発現抑制である．その中間産物であるShort interfering RNA（siRNA）は哺乳動物細胞においても，インターフェロン反応などの副反応なく目的の遺伝子を切断でき，治験段階に入っているアンチセンス核酸やライボザイムより，有効性，配列特異性いずれもはるかに優れている．その核酸医薬として臨床応用は特にウイルス性疾患において進行している．OFF-Target効果やデリバリー方法などまだまだ解決すべき問題点も多いが，siRNAの高い可能性から種々の方面において医療分野への応用が急速に進展していくことは間違いないものと思われる．

1. RNAiとは

今日までのウイルス増殖抑制の方法はワクチンかウイルス蛋白やウイルス特異酵素をターゲットとした創薬であった．ここ数年，ウイルスゲノム複製に関わる転写，翻訳を核酸レベルで直接抑制しようという試みが，アンチセンス核酸，ライボザイム，DNAエンザイムなどで行われていたが，十分な抑制効果は得られなかった．最近，新しい遺伝子発現抑制（gene silencing）として，これらをはるかに凌ぐ効果を持つRNA interference（RNAi）が注目されている．

長い2本鎖RNAiによって誘導される遺伝子発現抑制であるRNAi現象は植物から昆虫，哺乳動物にいたるまで広く保存して観察され，元来，真核細胞に備わった抗ウイルス機構として知られていた．細胞内に導入された2本鎖RNAはDicerと呼ばれるRNase III核酸分解酵素ファミリーによって21-24merの短い3'突出型の2本鎖RNAであるshort interference RNA（siRNA）に分解される．siRNAはアンチセンス鎖のみがとくほぐされて，ヘリケースなどの蛋白質から成るRNA蛋白質複合体であるRISC複合体（RNA-induced silencing complex）に取り込まれ，アンチセンス鎖に相補的な配列を持つターゲットRNAをその中央で分解する．しかし，哺乳動物における2本鎖RNAの導入はPKRや2' 5' oligosynthetaseの活性化による非特異的な翻訳抑制やRNAの分解が生じ，ホストの細胞の死んでしまうため，分子生物学的手法としても遺伝子治療の方法としてもRNAiの利用の大きな妨げになっていた．しかし，2000年に，RNAi機構の中間産物であるsiRNAを合成して用いることによってこれらの副反応が回避され，siRNAの配列に特異的な遺伝子発現抑制が可能となった[1]．さらに，siRNA配列を短い9merのループ配列でつないだstem型のパリンドロミックなsiRNA配列をpol III系のプロモーター下に挿入したsiRNA発現DNAプラスミドも開発された[2]．また，近年，加えてsiRNAの新たな遺伝子発現抑制機構としてsiRNAを介したDNAの主にプロモーター領域のCpGアイランドをターゲットにメチル化が転写抑制を起こすことも明らかになっている[3]．

RNAi機構は酵母からヒトに至るまで多くの生物種で保存されていて，その生物学的な意義としてはウィルスなどに対する防御機構として進化してきたという仮説が提唱されている．siRNAの発見以来，すぐにいくつかのウイルスにおいて，細胞内でのウイルス遺伝子の切断やウイルス遺伝子複製モデルにおいてsiRNAが有効であるとの報告が相次いでいる．現在まで，エイズウイルス（HIV）[4]，C型[5-8]・B型[9,10]肝炎ウイルス，ポリオウイルス[11]，インフルエンザウイルス[12,13]，ウエストナイルウイルス[13] SARSウイルス，ウエストナイルウイルスを含むの多くウイルスで

連絡先
〒113-8519　東京都文京区湯島1-5-45
TEL：03-5803-5234
FAX：03-5803-0169
E-mail：tak-yokota.nuro@tmd.ac.jp

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

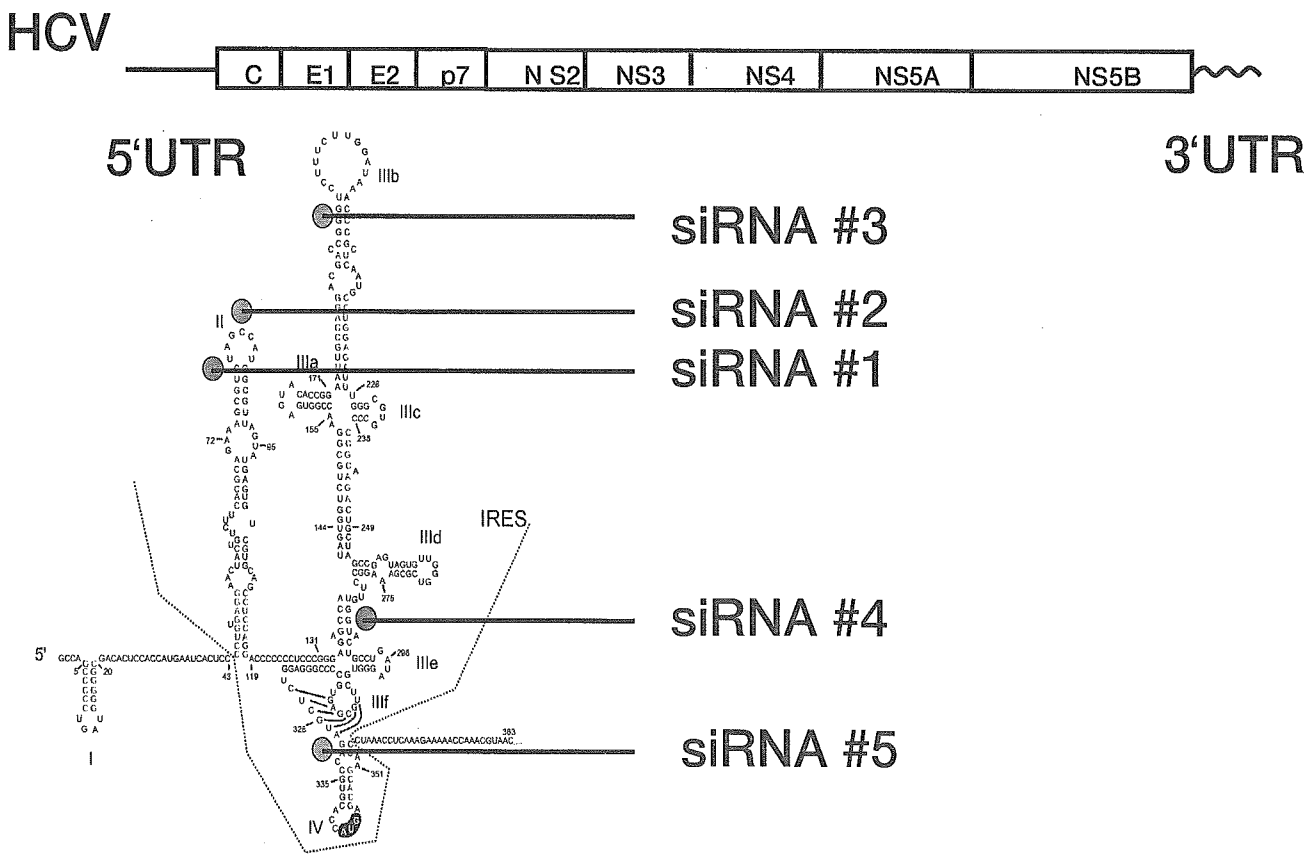


図1 （上）HCVの遺伝子構造と
（下）HCV 5'UTR IRESのRNAの2次構造とsiRNAのターゲット部位

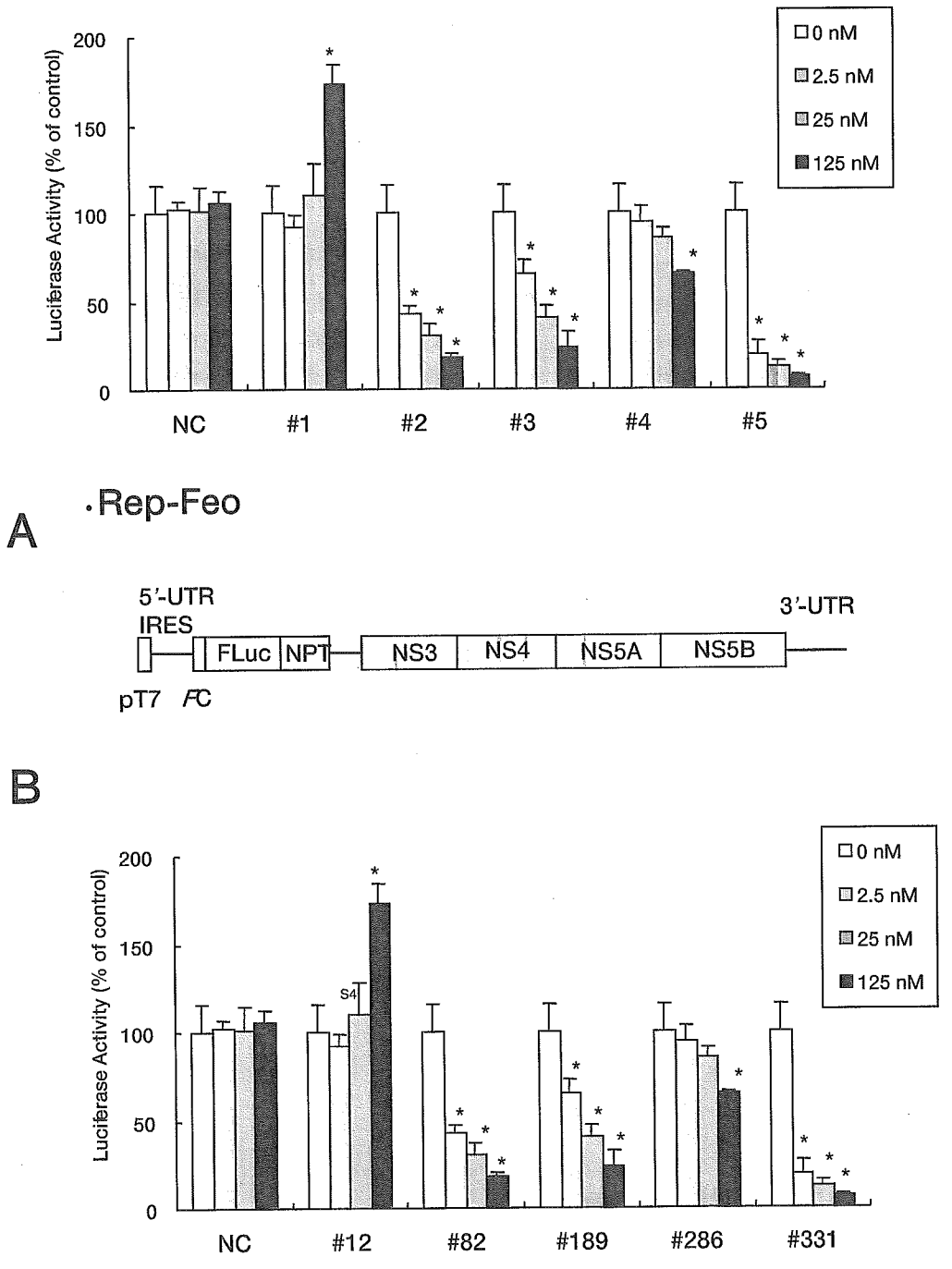


図 2　合成 siRNA の HCV レプリコン（Rep-Feo）へのルシフェラーゼ活性抑制効果（文献 5 から転載）

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

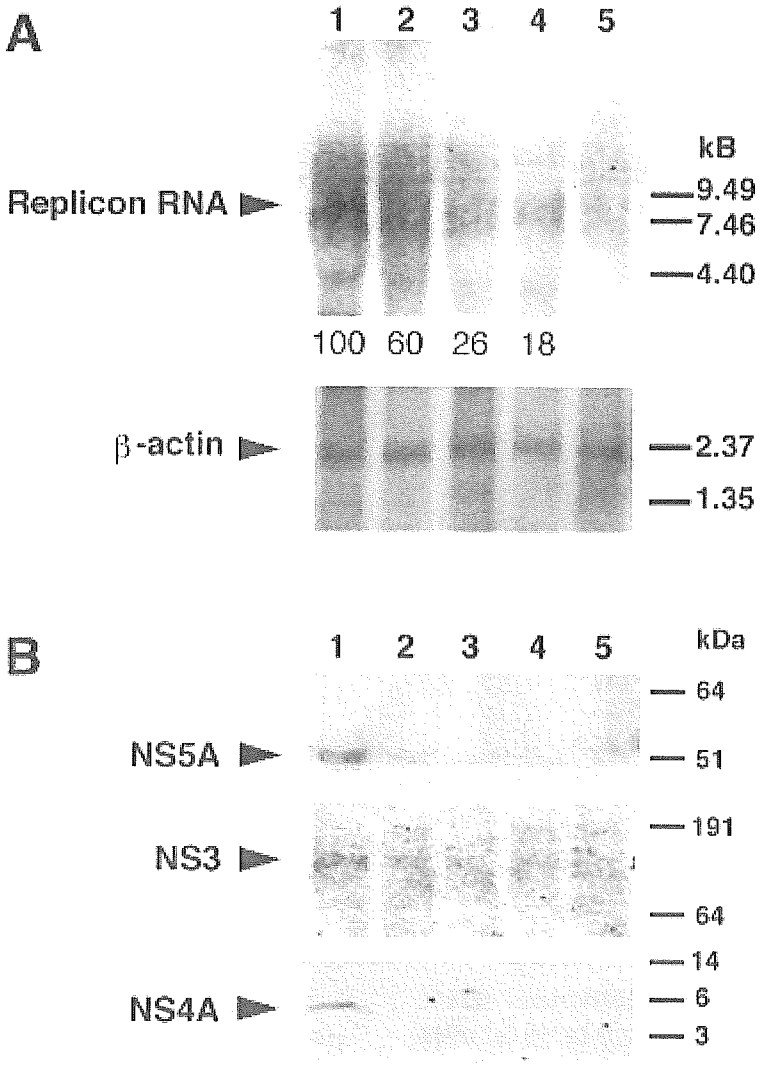


図3 siRNA331のHCVレプリコンRNA，非構造タンパクへの発現抑制効果（文献5から転載）
(A) レプリコン配列全長に対するプローブを用いたノザンブロット
(B) NS5Bに対するプローブや抗体を用いたウエスタンブロット
lane 1, mock; lane 2 siRNA 331 2.5 nM; lane 2 siRNA 331 25 nM; lane 2 siRNA 331 125 nM; no transfection

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．

文　献

1）Elbashir S., Harborth, Lendeckel W, Yalcin A, Weber K, Tuschl T.：Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411: 494-498, 2001.
2）BrummelkampTR, Bernards R, Agami R.：A system for stable expression of short interfering RNAs in mammalian cells. Science 29: 550-553, 2002.
3）Kawasaki H, Taira K.：Induction of DNA methylation and gene silencing by short interfering RNAs in human cells. Nature. 431: 211-217. 2004.
4）Kitabwalla MN, Ruprecht RM.：RNA interference--a new weapon against HIV and beyond. Engl. J. Med., 347: 1364-1367, 2002.
5）Yokota T, Sakamoto N, Enomoto N, et al. Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. EMBO Rep 4: 602-8, 2003.
6）Randel G, Grakoui A, Rice CM. Clearance of replicating hepatitis C virus replicon RNAs in cell culture by small intering RNAs Proc Natl Acad Sci USA 100: 235-340, 2003.
7）Kapadia SB, Brideau-Andersen A, Chisari FV. Interference of hepatitis C virus RNA replication by short interfering RNAs. Proc Natl Acad Sci USA. 100: 2014-8, 2003.
8）Wilson JA, Jayasena S, Khvorova A, et al. RNA interference blocks gene expression an RNA synthesis from hepatitis C replicons propagated in human liver cells. Proc Natl Acad Sci U S A. 100: 2783-8, 2003.
9）Shlomai A, Shaul Y.：Inhibition of hepatitis B virus expression and replication by RNA interference. Hepatology 37: 764-770, 2003.
10）Giladi H, Ketzinel-Gilad M, Rivkin L, Felig Y, Nussbaum O, Galun E.：Small interfering RNA inhibits hepatitis B virus replication in mice. Mol. Ther, 8: 769-776, 2003.
11）Gitlin, L, Karelsky S, Andino, R.：Short interfering RNA confers intracellular antiviral immunity in human cells. Nature, 418: 430-434, 2002.
12）Ge Q, McManus MT, Nguyen T, Shen CH, Sharp PA, Eisen, HN, Chen J.：RNA interference of influenza virus production by directly targeting mRNA for degradation and indirectly inhibiting all viral RNA transcription. Proc. Natl. Acad. Sci. U S A, 100: 2718-2723. 2003
13）McCown M, Diamond MS, Pekosz A.：The utility of siRNA transcripts produced by RNA polymerase i in down regulating viral gene expression and replication of negative- and positive-strand RNA viruses. Virology, 313: 514-524, 2003.
14）Amarzguioui, M., Holen, T., Babaie, E., & Prydz, H. Tolerance for mutations and chemical modifications in a siRNA, Nucleic Acids Res., 31: 589-595, 2003.
15）Boden, D. Oliver Pusch, Frederick Lee, Lynne Tucker, and Bharat Ramratnam Human Immunodeficiency

Virus Type 1 Escape from RNA Interference J. Viol., 77: 11531-11535, 2003.
16) Khvorova A, Reynolds A, Jayasena SD. Functional siRNAs and miRNAs exhibit strand bias. Cell. 115: 209-16. 2003.
17) Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, Zamore PD. Asymmetry in the assembly of the RNAi enzyme complex. Cell 115: 199-208, 2003.
18) Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorova A. : Rational siRNA design for RNA interference. Nat Biotechnol. 22: 326-330. 2004.
19) Garrus, J.E., von Schwedler, U.K., Pornillos, O.W., Morham, S.G., Zavitz, K.H., Wang, H.E., Wettstein, D.A., Stray, K.M., Cote, M., Rich, R.L., Myszka, D.G., & Sundquist, W.I. Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. Cell 107: 55-65, 2001
20) Surabhi RM, Gaynor RB. : RNA interference directed against viral and cellular targets inhibits human immunodeficiency Virus Type 1 replication. J Virol. 76: 12963-12973, 2002
21) Arteaga HJ, Hinkula J, van Dijk-Hard I, Dilber MS, Wahren B, Christensson B, Mohamed AJ, Smith CI. : Choosing CCR5 or Rev siRNA in HIV-1. Nat Biotechnol. 21: 230-231, 2003.
22) Qin XF, An DS, Chen IS, Baltimore, D. : Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. Proc Natl Acad Sci U S A, 100: 183-188, 2003
23) Miyagishi M, Sumimoto H, Miyoshi H, Kawakami Y, Taira K. : Optimization of an siRNA-expression system with an improved hairpin and its significant suppressive effects in mammalian cells. J Gene Med 6: 715-723, 2004.
24) McCaffrey AP, Meuse L, Pham, TT, Conklin D, Hannon G.J, Kay MA. : RNA interferece in adult mice. Nature 418: 38-39, 2002.
25) Lewis DL, Hagstrom JE, Loomis AG, Wolff JA, Herweijer H. Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. Nat Genet 32: 107-108. 2002.
26) Song E, Lee S-K, Wang J, Ince N, Ouyang N, Min J, Chen J, Shankar P, Lieberman J. : RNA interference targeting Fas protects mice from fulminant hepatitis. Nature Med 9: 347-351, 2003.
27) Zender L, Hutker S, Liedtke C, Tillmann HL, Zender S, Mundt B, Waltemathe M, Gosling T, Flemming P, Malek NP, Trautwein C, Manns MP, Kuhnel F, Kubicka S. : Caspase 8 small interfering RNA prevents acute liver failure in mice.Proc Natl Acad Sci U S A. 100: 7797-802, 2003
28) Soutschek J, Akinc A, Bramlage B, Charisse K, Constien R, Donoghue M, Elbashir S, Geick A, Hadwiger P, Harborth J, John M, Kesavan V, Lavine G, Pandey RK, Racie T, Rajeev KG, Rohl I, Toudjarska I, Wang G, Wuschko S, Bumcrot D, Koteliansky V, Limmer S, Manoharan M, Vornlocher HP. : Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. Nature 432: 173-8, 2004
29) Yano J, Hirabayashi K, Nakagawa S, Yamaguchi T, Nogawa M, Kashimori I, Naito H, Kitagawa H, Ishiyama K, Ohgi T, Irimura T. : Antitumor activity of small interfering RNA/cationic liposome complex in mouse models of cancer. Clin Cancer Res. 10: 7721-6, 2004
30) Spagnou S, Miller AD, Keller M. : Lipidic carriers of siRNA: differences in the formulation, cellular uptake, and delivery with plasmid DNA. Biochemistry. 43: 13348-56, 2004.
31) Xia H. Mao Q, Paulson HL, Davidson BL : . siRNA-mediated gene silencing in vitro and in vivo. Nat. Biotechnol., 20: 1006-1010, 2002.
32) Qin XF, An DS, Chen IS, Baltimore D. : Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. Proc. Natl. Acad. Sci. USA, 100: 183-188, 2003.
33) Barton GM, Medzhitov R. : Retroviral delivery of small interfering RNA into primary cells. Proc. Natl .Acad. Sci. USA. 99: 14943-14945, 2002.
34) Hommel JD, Sears R., Georgescu D, Simmons DL, DiLeone RJ. : Local gene knockdown in the brain using viral-mediated RNA interference. Nature Medicine 9: 1539 - 1544, 2003.
35) Nakai H, Fuess S, Storm TA, Muramatsu S, Nara Y, Kay MA. : Unrestricted hepatocyte transduction with adeno-associated virus serotype 8 vectors in mice. J Virol. 79: 214-24, 2005.
36) Wang Z, Zhu T, Qiao C, Zhou L, Wang B, Zhang J, Chen C, Li J, Xiao X. : Adeno-associated virus serotype 8 efficiently delivers genes to muscle and heart. Nat Biotechnol. 23: 321-8, 2005.

pp.1-8, 2005]

Gene therapy of virus replication with RNAi

Takanori Yokota

Department of Neurology and Neurological Science, Tokyo Medical and Dental University

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.

*si*RNA becomes *s*mart and *i*ntelligent

Makoto Miyagishi & Kazunari Taira

Designing small interfering RNAs is facilitated by an artificial neural network algorithm.

In just a few years, small interfering RNA (siRNA) has emerged as a powerful technology to specifically knockdown genes in mammalian cells both for functional analysis and for therapeutic purposes[1]. The success of this approach, also called RNA interference (RNAi), hinges on the affinity of an siRNA molecule for its target mRNA. Many different algorithms for designing siRNAs have been developed, but they remain of limited utility. In this issue, Hall *et al.*[2] report the first artificial neural network (ANN)-based algorithm for designing siRNAs and use it to generate a library of siRNAs for more than 24,000 human genes. The algorithm may enable one to design highly specific and efficient siRNAs for any organism, thus providing a powerful tool for RNAi.

RNAi proceeds through several steps. First, siRNAs transfected into cells are incorporated into the RNA-induced silencing complex (RISC), after or concurrent with the unwinding of double-stranded RNA. The RISC/siRNA complex then recognizes an mRNA sequence that is complementary to the antisense strand of the siRNA. Finally, the RISC enzymatic machinery cleaves the target mRNA, resulting in gene silencing. The efficiency of the cleavage step depends strongly on the chosen mRNA target sequence. For example, if one chooses random target sites, only 10% to 30% of siRNAs will show highly suppressive effects.

Many attempts have been made to understand the rules governing the silencing efficiency of siRNA. In an early report by Holen *et al.*[3], experiments with a set of siRNAs 'walking' across the target mRNA indicated that siRNA activity depends on GC content and that a difference of as little as 2 base pairs could markedly affect siRNA activity. Reynolds *et al.*[4] carried out a more detailed analysis using 120 siRNAs directed against two genes and identified several parameters: GC content, base preference at specific nucleotides, especially positions **1**, 7, **10** and 17 of the antisense strand (bold signifies a highly significant effect) and internal stability of the 3′ terminus of the sense strand. Khvorova *et al.*[5] and Schwarz *et al.*[6] subsequently suggested that the difference in internal stabilities between the 3′ and the 5′ termini, which determines the direction of siRNA unwinding and the incorporation of the guide strand into RISC, is also critical for siRNA activity. Finally, other groups have reported base preferences at positions 1, 4, 7, 9, 10, 14 and 19 of the antisense strand.

However, some of these parameters, especially base preferences with low significance,

Makoto Miyagishi & Kiazunari Taira are in the Department of Chemistry and Biotechnology, School of Engineering, University of Tokyo, Tokyo 113-8656, Japan and at the Gene Function Research Center, National Institute of Advanced Industrial Science and Technology, Tsukuba 305-8562, Japan.
e-mail: makoto-m@chembio.t.u-tokyo.ac.jp; taira@chembio.t.u-tokyo.ac.jp

are not consistent across the studies. This is most likely because the number of experimental siRNA data sets was often too small to identify weak correlations with any confidence. Combining data sets from different assays, and/or merging data sets that were measured with different target genes and reflect the different stabilities and concentrations of the siRNAs used, would not improve the quality of data either. All these limitations have made it difficult to identify the parameters that determine siRNA efficiency and to create reliable algorithms for designing siRNA.

Hall *et al.* began by assaying siRNA activity using a sophisticated fluorescent reporter system. Target cDNAs were inserted into the 3′ untranslated region of the enhanced yellow fluorescent protein (eYFP) mRNA, and a human cell line with siRNAs targeted against the corresponding mRNAs was transiently cotransfected with the resulting plasmids. The suppression of eYFP fluorescence induced by these siRNAs was measured and standardized against the fluorescence levels of enhanced cyan fluorescent protein expressed from the same reporter plasmid. Suppression by siRNA targeted against eYFP and by negative-control siRNAs targeted against an unrelated gene served to normalize siRNA transfection efficiency and to control for nonspecific inhibition of eYFP, respectively. The siRNA effects measured in the transient assay correlated well with the siRNA effects for the corresponding endogenous genes. The authors used this system to obtain high-quality data on about 3,000 siRNAs.

The authors then proceeded to analyze the 200 most potent and the 200 least potent siRNAs to elucidate base preferences. They found a few novel base preferences (positions 2(U), 11(U), 21(G) in the antisense strand) as well as several previously reported base preferences (positions 1(A or U), 7(U), 10(A), 19(C) in the antisense strand). One interesting result pertains to the G at position 21, which corresponds to the 3′ overhang of the antisense strand: the authors postulate that this base may be critical for mRNA recognition by the RNAi machinery.

To make an algorithm that can predict the silencing activity of siRNA from sequence information, Hall *et al.* chose to use an ANN-based approach. ANN is an information processing system originally inspired by the neural network architecture of the brain. It is capable of optimizing complex nonlinear

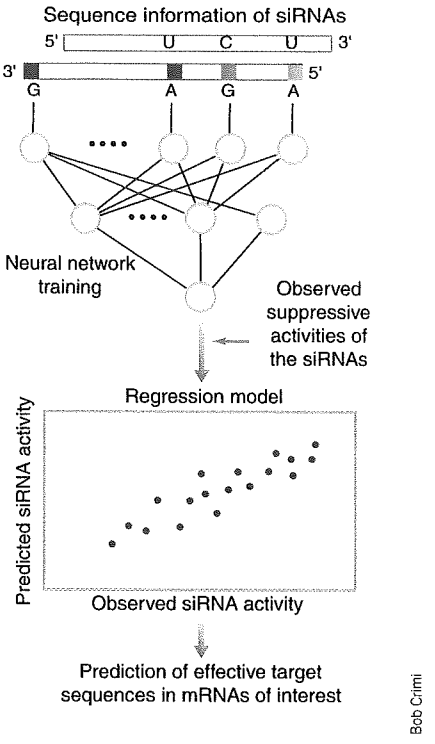


Figure 1 Schematic representation of an artificial neural network (ANN)-based approach for training, modeling and predicting siRNA efficiency.

relationships and has therefore been used for many applications, including classification, modeling and pattern recognition (**Fig. 1**).

Before building an ANN algorithm, the authors randomly divided the data into a training set (2,182 data points) and a testing set (249 data points). The ANN algorithm trained on the training set could predict the silencing activity of the testing set at a Pearson coefficient of correlation $r = 0.66$. The trained algorithm was also used to select effective siRNAs for three endogenous human genes.

When using ANN, as well as other regression methods, one of the most critical problems is the potential for 'overfitting.' If overfitting occurs as a result of 'overtraining' the algorithm, the error on the training set is forced to become very small. Consequently, the apparent correlation for the training data set becomes very high, but when new data not used for the training are presented to the ANN, the error becomes large, that is, the predictive ability for the new data is poor. The algorithm developed by Hall *et al.* shows a correlation for the training data set of 0.67, which is about the same as that of the testing data set. This result indicates that their ANN is appropriately trained and that little or no overfitting has occurred.

Most of the conventional methods for designing siRNAs use the aforementioned correlated parameters and select siRNA sequences by simply determining the value of the appropriate parameters or by a score that is calculated by a linear summation of the weighted available parameters. However, these methods cannot handle complex sequence motifs and/or synergistic relations between two or more parameters. Indeed, we have identified synergistic relations among parameters, for example, internal stabilities between the 5′- and 3′-termini that significantly affect siRNA activity[7,8]. Conversely, to run ANN when synergistic and/or complex effects exist requires large and accurate data sets—in other words, success depends largely on the size and quality of the data sets used.

The correlation coefficient for the prediction of 0.66 in Hall *et al.*'s study might be sufficient for designing siRNAs for biological or clinical use, but it may not fully account for the physicochemical mechanism of siRNA. This implies the existence of other relevant factors beyond sequence information, such as RNA modifications and the cellular localization and secondary structure of target mRNA. Thus, there is likely room for improvement of Hall *et al.*'s already sophisticated algorithm. Such improvements promise even higher-quality, large-scale siRNA libraries, which will facilitate genome-wide approaches to biological questions in numerous living systems—approaches that have already proven very fruitful[9,10].

1. http://www.nature.com/focus/rnai/index.html
2. Hall, T. *Nat. Biotechnol.* **23**, 995–1001 (2005).
3. Holen, T., Amarzguioui, M., Wiiger, M.T., Babaie, E. & Prydz, H. *Nucleic Acids Res.* **30**, 1757–1766 (2002).
4. Reynolds, A. *et al. Nat. Biotechnol.* **22**, 326–330 (2004).
5. Khvorova, A., Reynolds, A. & Jayasena, S.D. *Cell* **115**, 209–216 (2003).
6. Schwarz, D.S. *et al. Cell* **115**, 199–208 (2003).
7. Miyagishi, M. & Taira, K. *Oligonucleotides* **13**, 325–333 (2003).
8. Taira, K. & Miyagishi, M. Japanese patent application 2003-349283 (2003).
9. Fraser, A. *Nature* **428**, 375–378 (2004).
10. Matsumoto, S., Miyagishi, M., Akashi, H., Nagai, R. & Taira, K. *J. Biol. Chem.* **280**, 25687–25696 (2005).

A20 Is a Negative Regulator of IFN Regulatory Factor 3 Signaling[1]

Tatsuya Saitoh,* Masahiro Yamamoto,† Makoto Miyagishi,§¶ Kazunari Taira,§¶ Makoto Nakanishi,‖ Takashi Fujita,# Shizuo Akira,†‡ Naoki Yamamoto,* and Shoji Yamaoka[2]*

IFN regulatory factor 3 (IRF-3) is a critical transcription factor that regulates an establishment of innate immune status following detection of viral pathogens. Recent studies have revealed that two IκB kinase (IKK)-like kinases, NF-κB-activating kinase/Traf family member-associated NF-κB activator-binding kinase 1 and IKK-*i*/IKKε, are responsible for activation of IRF-3, but the regulatory mechanism of the IRF-3 signaling pathway has not been fully understood. In this study, we report that IRF-3 activation is suppressed by A20, which was initially identified as an inhibitor of apoptosis and inducibly expressed by dsRNA. A20 physically interacts with NF-κB-activating kinase/Traf family member-associated NF-κB activator-binding kinase 1 and IKK-*i*/IKKε, and inhibits dimerization of IRF-3 following engagement of TLR3 by dsRNA or Newcastle disease virus infection, leading to suppression of the IFN stimulation response element- and IFN-β promoter-dependent transcription. Importantly, knocking down of A20 expression by RNA interference results in enhanced IRF-3-dependent transcription triggered by the stimulation of TLR3 or virus infection. Our study thus demonstrates that A20 is a candidate negative regulator of the signaling cascade to IRF-3 activation in the innate antiviral response. *The Journal of Immunology,* **2005, 174: 1507–1512.**

The innate immune system is an important, evolutionarily conserved mechanism that confers host defense against viral and microbial infection (1–3). IFN regulatory factor 3 (IRF-3)[3] is a ubiquitously expressed transcription factor that regulates primary induction of type I IFN, IFN-αβ, and plays a critical role for establishing innate immune status in response to invasion of pathogens (1–2, 4). Although IRF-3 is retained in the cytoplasm of unstimulated cells, it is phosphorylated and forms a dimer upon viral infection, which then translocates to the nucleus, binds to IFN stimulation response element (ISRE) and enhances the transcription of a set of genes including IFN-β (2, 4, 5). Recent studies revealed that two noncanonical IκB kinase (IKK)-like kinases, NF-κB-activating kinase (NAK)/Traf family member-associated NF-κB activator-binding kinase 1 (TBK1) and IKK-*i*/IKKε, could induce the dimerization of IRF-3 by enhancing phosphorylation of IRF-3 and play essential roles for IRF-3-dependent transcriptional activation (6, 7).

Viral and microbial pathogens can be detected by TLR3 and TLR4, which recognize viral dsRNA and bacterial LPS, respectively (3, 8). Engagement of these TLRs triggers the IRF-3 and NF-κB signaling pathways and confers the rapid induction of IFN-β. Although MyD88 is a common adaptor protein for TLRs and plays important roles for NF-κB activation, TLR3 and TLR4 still mediate both IRF-3 and NF-κB activation in MyD88-deficient mice, indicating that the MyD88-independent signaling pathways for production of IFN-β are triggered by engagement of these TLRs (3, 8). We and others identified adaptor molecules, Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF) (also called TICAM-1) and TRIF-related adaptor molecule (TRAM; also called TICAM-2 or TIRP) as mediators in the MyD88-independent signaling pathways (8–11). TRAM interacts with TLR4 and is specifically involved in TLR4-induced IRF-3 activation. TRIF, which interacts with TLR3 and TRAM, is involved in both TLR3- and TLR4-mediated signaling pathways and associates with NAK/TBK1 to initiate IRF-3 activation (12).

Although recent discoveries identified critical mediators of IRF-3 signaling, regulatory mechanisms of these molecules are not fully understood. Because NAK/TBK1 and IKK-*i*/IKKε are IKK-like molecules, we hypothesized that the regulatory mechanism of IRF-3 kinases might be similar to that of the canonical IKKα/IKKβ/NF-κB essential modulator (NEMO) complex. We therefore examined regulators of the IKK complex activation for participation in IRF-3 signaling pathways and have found that A20 potently inhibits IRF-3 activation. A20 is a 90-kDa protein whose expression is induced by a variety of stimuli including poly(I)·poly(C) (polyIC), LPS, and TNF-α (13, 14). A recent study indicated that viral infection also induced A20 expression through activation of protein kinase R (15). A20 has a deubiquitinase domain at the N terminus and seven repeats of zinc finger domain at the C terminus, although the role of these domains for A20 function is not fully understood (13, 16). A recent study using mice deficient in A20

*Department of Molecular Virology, Graduate School of Medicine, Tokyo Medical and Dental University, Tokyo, †Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Osaka, ‡Exploratory Research for Advanced Technology, Japan Science and Technology Agency, Osaka, §Department of Chemistry and Biotechnology, School of Engineering, The University of Tokyo, Tokyo, ¶National Institute of Advanced Industrial Science and Technology, Gene Function Research Center, Tsukuba Science City, ‖Department of Biochemistry, Graduate School of Medicine, Nagoya City University, Nagoya, Aichi, and #Department of Tumor Cell Biology, The Tokyo Metropolitan Institute for Medical Science, Tokyo, Japan

Received for publication May 13, 2004. Accepted for publication September 17, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by a grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to N.Y. and S.Y.), and by a grant-in-aid for Research Fellow of Japan Society for the Promotion of Science (to T.S.).

[2] Address correspondence and reprint requests to Dr. Shoji Yamaoka, Department of Molecular Virology, Graduate School of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan. E-mail address: shojmmb@tmd.ac.jp

[3] Abbreviations used in this paper: IRF, IFN regulatory factor; ISRE, IFN stimulation response element; IKK, IκB kinase; NAK, NF-κB-activating kinase; TBK1, Traf family member-associated NF-κB activator-binding kinase 1; TRIF, Toll/IL-1R domain-containing adaptor inducing IFN-β; TRAM, TRIF-related adaptor molecule; NEMO, NF-κB essential modulator; polyIC, poly(I)·poly(C); NDV, Newcastle disease virus; siRNA, small inhibitory RNA; TRAF, TNFR-associated factor.

0022-1767/05/$02.00

provided genetic evidence that A20 worked as a negative regulator in NF-κB signaling pathways. Injection of TNF-α in A20-deficient mice induced severe inflammation due to persistent activation of NF-κB (17). These mice exhibited elevated sensitivity to endotoxin shock, suggesting that A20 might negatively regulate TLR-initiated signaling pathways (17, 18). However, involvement of A20 in IRF-3 signaling pathways triggered by engagement of TLR or virus infection has not been reported.

In this study, we demonstrate that A20 interacts with IRF-3 kinases, NAK/TBK1 and IKK-*i*/IKKε, and inhibits TLR3- or virus-induced IRF-3 dimerization and ISRE-dependent transcriptional activation.

Materials and Methods

Reagents

Monoclonal anti-HA Ab (12CA5) was a kind gift from Dr. A. Israël (Institut Pasteur, Paris, France). Monoclonal anti-FLAG Ab (M2) and anti-HA Ab (HA-7) were purchased from Sigma-Aldrich. Anti-actin Ab (C-2) and anti-IRF-3 Ab (FL-425) were purchased from Santa Cruz Biotechnology. Polyclonal anti-human IRF-3 (phospho-Ser^{386}) Ab was described previously (5). PolyIC was purchased from Amersham Biosciences. Newcastle disease virus (NDV) was prepared as described previously (19). All other regents were purchased from Sigma-Aldrich unless otherwise noted.

Plasmids

pcDNA3-HA-A20, pFLAG-CMV-1-hTLR3, pEF1-*lacZ*, and p125-luc were kind gifts from Drs. D. Wallach (The Weizmann Institute of Science, Rehovot, Israel), K. Fitzgerald (University of Massachusetts Medical School, Worcester, MA), S. Memet (Institut Pasteur, Paris, France), and T. Taniguchi (University of Tokyo, Tokyo, Japan), respectively. pcDNA3 and pISRE-luc were purchased from Invitrogen Life Technologies and Stratagene, respectively. pcDNA3-FLAG-NAK, pcDNA3-FLAG-dnNAK (K38A), pEF-FLAG-IKK-*i*, pEF-HA-IRF-3, pEF-HA-dnIRF-3 (58–427), and pEF-p50-IRF-3 5D were described previously (5, 12, 19, 20). Complementary DNAs encoding either the N-terminal 378 aa (1–378) or C-terminal 412 aa (379–790) of human A20 were amplified by PCR using pcDNA3-HA-A20 as a template and inserted into pcDNA3-HA. The resultant plasmids were referred to as pcDNA3-HA-A20-N or pcDNA3-HA-A20-C, respectively. The primer sequences used for PCR are available upon request. pU6 plasmids capable of small inhibitory RNA (siRNA) expression were constructed according to the procedures described previously (21). Sequences inserted immediately downstream of U6 promoter were as follows (only sense sequence is shown): specific to A20, 5′-GGAAACAGACACACGCAAC-3′; the unrelated control, 5′-GTAGCGCGGTGTATTATAC-3′. The resultant plasmids were referred as pU6-A20i or pU6-Ctli, respectively.

Cell culture, transfection, and reporter assay

293/TLR3 cells were described previously (22). The 293, 293/TLR3, and HeLa cells used in this study were all maintained in DMEM supplemented with 10% FCS, 100 U/ml penicillin G, and 100 μg/ml streptomycin. THP-1 cells were maintained in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin G, and 100 μg/ml streptomycin. 293 and 293/TLR3 cells were transfected by the calcium-phosphate method as described previously (23). HeLa cells were transfected by FuGene6 transfection reagent (Roche) according to the manufacturer's instructions. THP-1 cells were transfected by DMRIE-C transfection reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. 293, 293/TLR3, and HeLa cells were transfected with 250 ng of EF1-*lacZ* and 250 ng of pISRE-luc or p125-luc along with the indicated effector plasmid. THP-1 cells were transfected with 500 ng of EF1-*lacZ* and 500 ng of pISRE-luc along with the indicated effector plasmid. After the transfection, cells were treated as indicated and lysed in lysis buffer (25 mM Tris-HCl, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, 15% glycerol). Luciferase activity was normalized based on β-galactosidase activity. All of the experiments were conducted at least six times. The results were essentially reproducible.

Preparation of whole-cell extracts, immunoblotting, and immunoprecipitation

Cells were suspended in lysis buffer (20 mM HEPES (pH 7.6), 150 mM NaCl, 0.5 mM EDTA, and 0.1% Nonidet P-40) supplemented with 1 mM

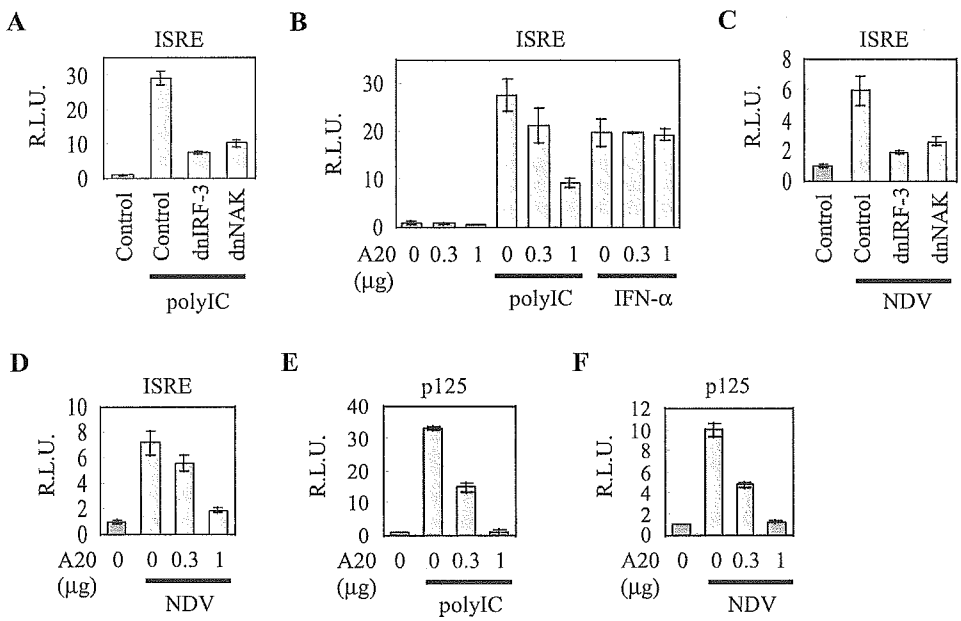


FIGURE 1. A20 suppresses IRF-3-dependent transcription. *A* and *B*, 293/TLR3 cells were transfected with 1 μg of the indicated expression plasmid (*A*) or the indicated amounts of pcDNA3-HA-A20 (*B*) along with pISRE-luc and pEF1-*lacZ*. Total amount of effecter plasmid (1 μg) was kept constant by addition of pcDNA3. Twenty-four hours after transfection, the cells were stimulated with polyIC (10 μg/ml) or IFN-α (2000 U/ml) for 3 h. *C* and *D*, 293 cells were transfected with 1 μg of the indicated expression plasmid (*C*) or the indicated amounts of pcDNA3-HA-A20 (*D*) along with pISRE-luc and pEF1-*lacZ*. Total amount of effecter plasmid (1 μg) was kept constant by addition of pcDNA3. Twenty-four hours after transfection, the cells were infected with NDV for 12 h. *E* and *F*, 293/TLR3 cells or 293 cells were transfected with the indicated amounts of pcDNA3-HA-A20 along with p125-luc (IFN-β promoter) and pEF1-*lacZ*, and then the cells were stimulated with polyIC for 3 h (*E*) or infected with NDV (*F*) for 12 h, respectively. The harvested cells were subjected to luciferase reporter assays. The luciferase activity was normalized based on the β-galactosidase activity. The values shown are means ± SD from three separate transfections. R.L.U., Relative luciferase unit.

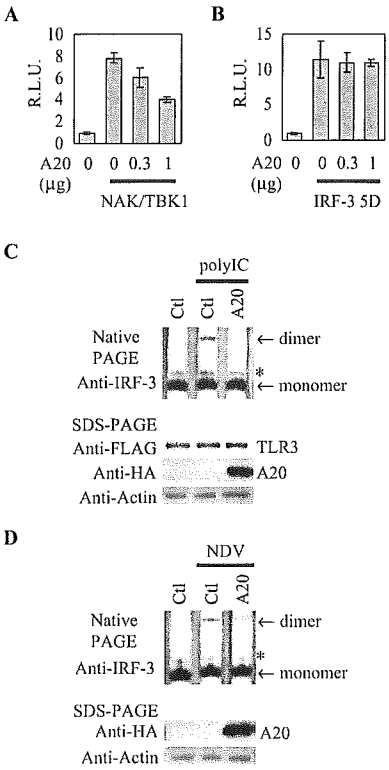


FIGURE 2. A20 inhibits dimerization of IRF-3. *A* and *B*, 293 cells were transfected with 50 ng of pcDNA3-FLAG-NAK (*A*) or 15 ng of pEF-p50-IRF-3 5D (*B*) along with the indicated amounts of pcDNA3-HA-A20, pISRE-luc, and pEF1-*lacZ* for 24 h. The harvested cells were subjected to luciferase reporter assays. *C*, 293 cells were transfected with 1 μg of pcDNA3 or pcDNA3-HA-A20 along with 100 ng of pFLAG-CMV-1-hTLR3. Twenty-four hours after transfection, the cells were stimulated with polyIC for 1 h. *D*, 293 cells were transfected with 1 μg of pcDNA3 or pcDNA3-HA-A20. Twenty-four hours after transfection, the cells were infected with NDV for 12 h. The whole-cell extracts were subjected to native PAGE or SDS-PAGE. The membranes were probed with the indicated Abs. The asterisks indicate nonspecific bands. Positions of IRF-3 monomer and dimer are indicated.

PMSF and 1 mM Na_3VO_4. Extracts were cleared by centrifugation. Immunoprecipitation and immunoblotting were performed as described previously (23). Briefly, cell lysates were incubated with anti-FLAG mAb (M2) for 1 h and then incubated with protein G-Sepharose for 1 h. The beads were washed four times with lysis buffer. Precipitated proteins and whole-cell extracts were fractionated by 8% SDS-PAGE, and transferred onto Immobilon membranes (Millipore), and blots were revealed with an ECL detection system (Amersham Biosciences). All of the experiments were conducted at least twice. The results were essentially reproducible.

Detection of IRF-3 dimer by native PAGE

Cells were suspended in lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1% Nonidet P-40) supplemented with 1 mM PMSF and 1 mM Na_3VO_4. Extracts were cleared by centrifugation. Whole-cell extracts (10 μg) were fractionated by 7.5% native PAGE, and transferred onto Immobilon membranes, and blots were revealed with an ECL detection system. All of the experiments were conducted at least twice. The results were essentially reproducible.

Results

A20 suppresses IRF-3 activation

We examined involvement of proteins associated with the IKKα/IKKβ/NEMO complex for IRF-3 regulation, because the NAK/TBK1 and IKK-*i*/IKKε are IKK-like molecules. We used two well-characterized stimuli to trigger IRF-3 signaling pathways, engagement of TLR3 with polyIC and infection with NDV, which indeed activated ISRE-dependent transcription in a manner that depends on NAK/TBK1 and IRF-3 (Fig. 1, *A* and *C*). As a result, we found that A20 inhibited TLR3 stimulation- or NDV infection-induced ISRE-dependent transcription in a dose-dependent manner (Fig. 1, *B* and *D*). In contrast, expression of A20 did not affect IFN-α-induced activation (Fig. 1*B*), indicating that A20 specifically regulates IRF-3 signaling. We next asked whether A20 modulates the activity of the IFN-β promoter, which harbors ISRE recognized by IRF-3. Transfection studies revealed that A20 potently inhibited TLR3- or NDV-induced IFN-β promoter activation (Fig. 1, *E* and *F*). A20 also inhibited NAK/TBK1-induced, but not the constitutively active IRF-3 5D mutant-induced ISRE-dependent transcription (Fig. 2, *A* and *B*). In addition, A20 inhibited the polyIC- or NDV-induced dimerization of endogenous IRF-3 (Fig. 2, *C* and *D*). These results strongly suggest that A20 acts on a common mediator(s) that regulates IRF-3 activation induced by TLR3 engagement or NDV infection.

A20 interacts with NAK/TBK1 and IKK-i/IKKε

Because NAK/TBK1 is a common regulator of TLR- or virus-induced IRF-3 activation, we next examined interaction of A20 with NAK/TBK1 and found that A20 was coimmunoprecipitated with NAK/TBK1 (Fig. 3*A*). In addition, A20 was associated with another IRF-3 kinase, IKK-*i*/IKKε (Fig. 3*B*). Interestingly, when A20 was cotransfected with NAK/TBK1 or IKK-*i*/IKKε, A20 was found to migrate more slowly in immunoblot studies (Fig. 3, *A* and *B*), as had been shown for TRIF, which associates with NAK/TBK1 (12). A20 has a deubiquitinase domain at the N terminus and repeated zinc finger domains at the C terminus (Fig. 3*C*). Previous studies demonstrated that TNFR-associated factor (TRAF)2 and TRAF6 interact with the N terminus of A20, whereas A20-binding inhibitor of NF-κB interacts with the C terminus of A20 (13). NEMO was reported to interact with both the N and C termini of A20 (24). To determine which functional domain(s) of A20 is required for the interaction with these kinases, we generated deletion constructs capable of expressing the N terminus (aa 1–378) or C terminus (aa 379–790) of A20 (Fig. 3*C*). Immunoblot analyses detected these mutants at expected positions, although the level of expression of the N terminus of A20 was higher than the others (Fig. 3*D*). Immunoblotting studies coupled with immunoprecipitation revealed that NAK/TBK1 and IKK-*i*/IKKε interacted with the C-terminal zinc finger and N-terminal deubiquitinase domains of A20 (Fig. 3, *E* and *F*).

Because A20 interacts with NAK/TBK1, which phosphorylates IRF-3, we examined whether A20 inhibits NAK/TBK1-induced IRF-3 phosphorylation. Immunoblotting with phosphospecific anti-IRF-3 Ab following native PAGE demonstrated that NAK/TBK1-induced phosphorylation of IRF-3 at Ser^{386}, one of the important residues for the dimerization of IRF-3 (5), was impaired in cells expressing the full length or C terminus of A20 (Fig. 3*G*, *upper panels*). In contrast, NAK/TBK1-induced phosphorylation of IRF-3 was not inhibited in cells expressing the N terminus of A20. As reported previously (7), slowly migrating, multiply phosphorylated species of IRF-3 were detected by SDS-PAGE followed by immunoblotting with phosphospecific or conventional anti-IRF-3 Abs when IRF-3 was cotransfected with NAK/TBK1, and these modifications were weakened by coexpression of the full length or C terminus of A20 (Fig. 3*G*, *lower panels*). We verified in transfection studies that the biochemical activity of each A20 domain correlated with the functional requirement for inhibition of TLR3- or NDV-induced ISRE-dependent transcription (Fig. 3, *H* and *I*). The C-terminal zinc finger domain of A20 suppressed ISRE-dependent transcription induced by TLR3 stimulation or

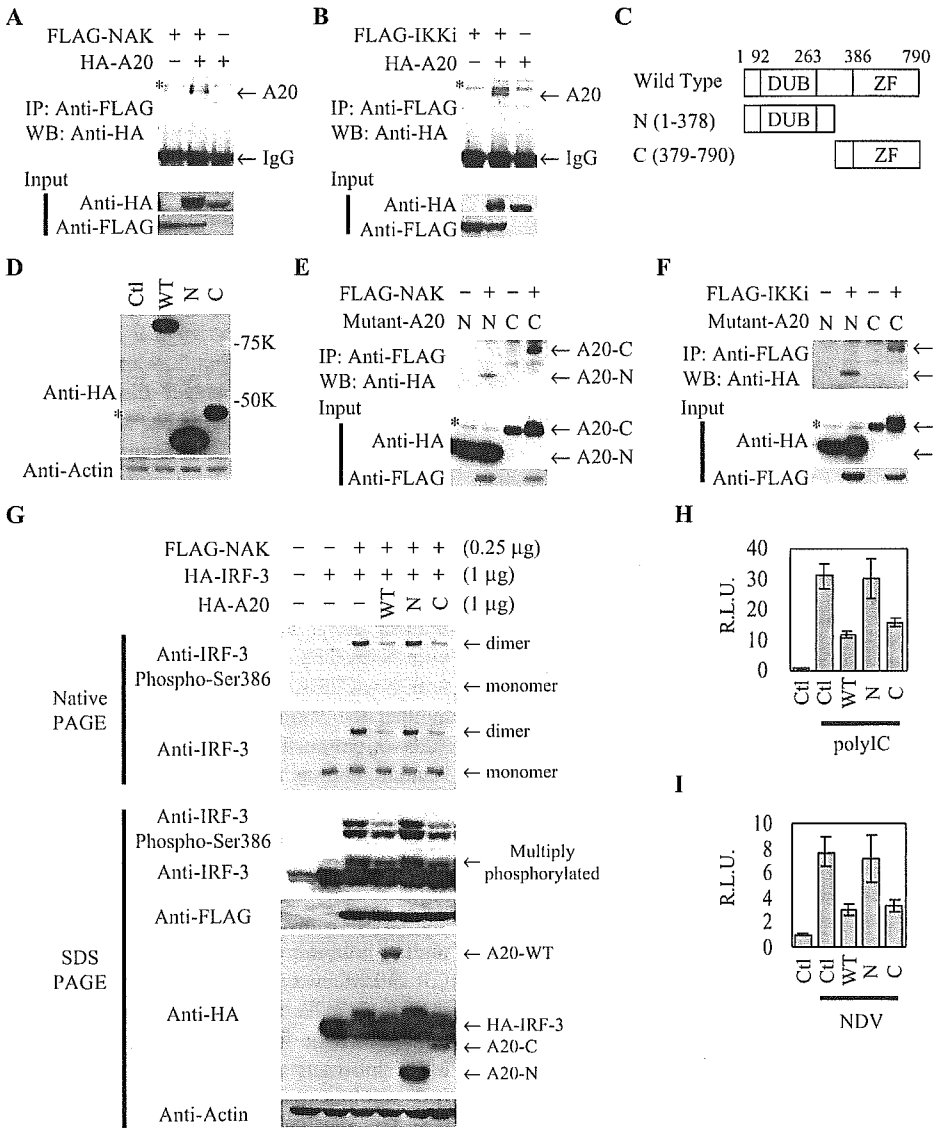


FIGURE 3. A20 interacts with NAK/TBK1 and IKK-*i*/IKKε. *A* and *B*, 293 cells were transfected with 5 μg of the indicated expression plasmids for 24 h. Total amount of plasmid (10 μg) was kept constant by addition of pcDNA3. The cell lysates were subjected to immunoprecipitation with anti-FLAG Ab followed by Western blotting with anti-HA and anti-FLAG Abs. IP, Immunoprecipitation; WB, Western blotting. The asterisks indicate nonspecific bands. *C*, Schematic representation of wild-type (WT) and mutant A20 proteins (N and C). The mutant N encodes the N-terminal 378 aa (1–378) and contains the deubiquitinase domain (DBU). The mutant C encodes the C-terminal 412 aa (378–790) and has seven zinc finger motifs (ZF). *D*, 293 cells were transfected with the indicated expression constructs. Cells extracts (10 μg) were subjected to Western blotting analysis with anti-HA and anti-actin Abs. Ctl, Control vector transfection. The asterisk indicates nonspecific bands. *E* and *F*, 293 cells were transfected with 5 μg of the indicated plasmids for 24 h. Total amount of plasmid (10 μg) was kept constant by addition of pcDNA3. The cell lysates were subjected to immunoprecipitation with anti-FLAG Ab followed by Western blotting with anti-HA and anti-FLAG Abs. The asterisks indicate nonspecific bands. *G*, 293 cells were transfected with indicated expression plasmids. Total amount of plasmid (2.25 μg) was kept constant by addition of pcDNA3. Twenty-four hours after transfection, whole-cell extracts were prepared and subjected to native PAGE or SDS-PAGE. Each membrane was sequentially probed with the indicated Abs. *H* and *I*, Reporter assays. 293/TLR3 cells (*H*) and 293 cells (*I*) were transfected with 1 μg of the indicated expression plasmids along with pEF1-*lacZ* and pISRE-luc, and then treated with polyIC for 3 h (*H*) or infected with NDV for 12 h (*I*), respectively. Ctl, Control vector transfection.

NDV infection as efficiently as the full-length A20, whereas the N terminus of A20 failed to do so.

siRNA to A20 enhances the signal-induced IRF-3-dependent transcription

To verify the role of endogenous A20, we generated a siRNA expression construct capable of knocking down the A20 expression. Expression of siRNA specific to A20 effectively reduced the level of transfected A20, whereas it did not affect that of IRF-3 (Fig. 4*A*). Expression of this siRNA specifically enhanced ISRE-dependent transcription induced by TLR3 stimulation or NDV infection, whereas it barely caused additional ISRE-dependent transcription in unstimulated 293 and 293/TLR3 cells or in 293/TLR3 cells stimulated with IFN-α (Fig. 4, *B* and *E*). Similar results were obtained with HeLa cells (Fig. 4*C*) and immunologically relevant THP-1 cells (*D*). Consistent with the ability of A20 to suppress NAK/TBK1-induced ISRE-dependent transcription (Fig. 2*A*), expression of siRNA to A20 enhanced this transcriptional activation (Fig. 4*F*). In contrast, it did not alter the constitutively active IRF-3 5D mutant-induced ISRE-dependent transcription (Fig. 4*G*). These results indicate that endogenous A20 negatively regulates IRF-3 signaling following initial stimulation of the system.

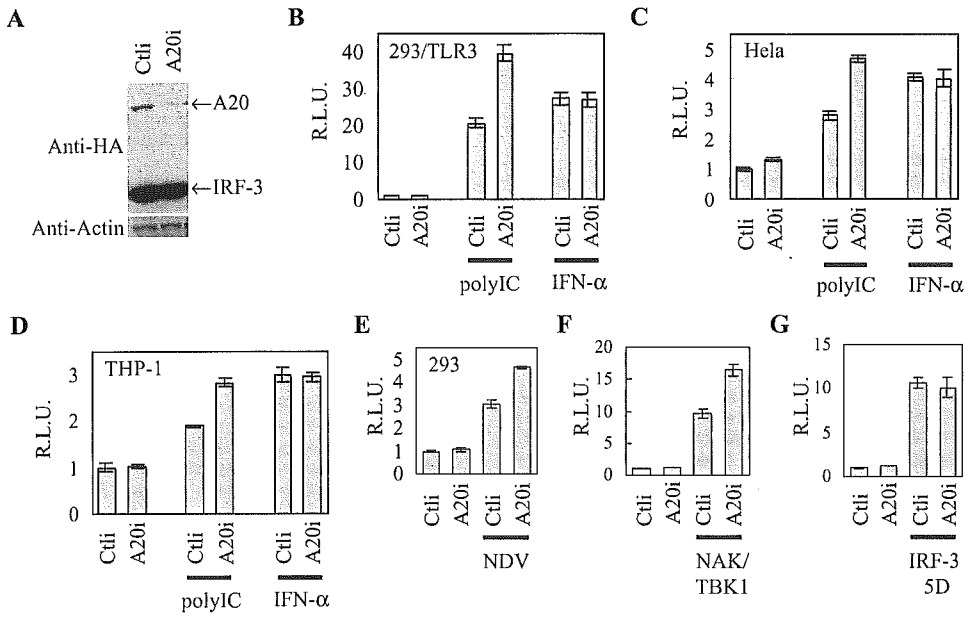


FIGURE 4. Suppression of A20 expression by RNA interference enhances the signal-induced IRF-3-dependent transcription. *A*, 293 cells were transfected with 500 ng of pU6-Ctli or pU6-A20i along with 100 ng of pcDNA3-HA-A20 and 100 ng of pEF-HA-IRF3. Cells extracts (10 μg) were subjected to Western blotting analysis with anti-HA and anti-actin Abs. Ctli, Control siRNA; A20i, siRNA specific to A20. *B* and *C*, 293/TLR3 cells (*B*) or HeLa cells (*C*) were transfected with 500 ng of pU6-Ctli or pU6-A20i along with pEF1-*lacZ* and pISRE-luc. Thirty-six hours after transfection, the cells were stimulated with polyIC or IFN-α for 12 h. *D*, THP-1 cells were transfected with 1 μg of pU6-Ctli or pU6-A20i along with pEF1-*lacZ* and pISRE-luc. Thirty-six hours after transfection, the cells were stimulated with polyIC or IFN-α for 8 h. *E*, 293 cells were transfected with 500 ng of pU6-Ctli or pU6-A20i along with pEF1-*lacZ* and pISRE-luc. Thirty-six hours after transfection, the cells were infected with NDV for 12 h. *F* and *G*, 293 cells were transfected with 50 ng of pcDNA3-FLAG-NAK (*F*) or 15 ng of pEF-p50-IRF-3 5D (*G*) along with indicated siRNA expression plasmid, pEF1-*lacZ* and pISRE-luc for 36 h. The harvested cells were subjected to luciferase reporter assay.

Discussion

Previous studies identified critical mediators in TLR signaling pathways that play important roles in innate immune responses. In addition, recent reports showing that mice deficient in suppressor of cytokine signaling-1, IL-1R-associated kinase M, or ST2, a negative regulator of NF-κB signaling, are hypersensitive to endotoxin shock, strongly suggest that negative feedback regulation of TLR signaling is important to protect the host from excessive immune response (25–28). Because IRF-3 is known to mediate endotoxin shock or virus-induced cell death, the IRF-3 activity should also be strictly controlled (29, 30). Several negative regulators of TLR-triggered NF-κB signaling were identified, but those of IRF-3 signaling have not been described except for IRF-2, which competes with IRF-3 for the recruitment of CREB binding protein (31). In this study, we have demonstrated for the first time a negative regulation of IRF-3 signaling triggered by TLR3 engagement or virus infection at the level of IRF-3 kinases. Because A20 was identified as a polyIC- or LPS-inducible protein (13–14), A20 might participate in the negative feedback regulation of IRF-3 signaling. Thus, our present study provides a molecular basis for a role of A20 in evasion of fatal excessive immune response in hosts suffering from virus infection. Pitha and colleagues (32) reported that pretreatment of cells with LPS impaired virus-induced phosphorylation and subsequent nuclear translocation of IRF-3. Because A20 is induced by LPS stimulation, our results that A20 interferes with the NAK/TBK1 and IKK-*i*/IKKϵ-mediated IRF-3 signaling could partly explain the impaired IRF-3 activation after LPS treatment. Studies using A20-deficient mice will clarify the roles of A20 for the negative regulation of antiviral innate immune responses in vivo.

We showed potent suppression of the IFN-β promoter by A20. This may partly be due to A20 inhibition of NF-κB activation (data not shown). Because A20 was reported to inhibit NF-κB activation through TRAF6 or receptor interacting protein, which transduce signals from TLR3-TRIF and interact with NEMO, it is reasonable to assume that A20 interferes with the TRAF6, receptor interacting protein, and NEMO signaling axis triggered by TLR3 stimulation or virus infection (12, 13, 24, 33, 34).

We have demonstrated the important role of the C-terminal zinc finger domain of A20 for inhibition of IRF-3 activation induced by TLR3 stimulation or NDV infection (Fig. 3, *H* and *I*). The C terminus of A20 interacts with NAK/TBK1 and inhibits NAK/TBK1-induced phosphorylation and subsequent dimerization of IRF-3 (Fig. 3*G*). In contrast, the N terminus of A20 does not inhibit NAK/TBK1-induced IRF-3 activation, although it also interacts with NAK/TBK1. These results indicate that A20 inhibits NAK/TBK1 from phosphorylating IRF-3, and that this inhibition cannot simply be explained by the binding of A20 or A20 mutant to NAK/TBK1. Beyaert and colleagues (33, 35) reported that the C terminus of A20 was also required for the inhibition of NF-κB activation, and that the zinc finger motifs of A20 compensate the function each other. However, the functional consequences of the A20 C terminus remain to be clarified, and further study will be required to define the molecular mechanism responsible for the inhibition of IRF-3 activation by A20.

Recently, several proteins that contain A20-like functional domain were reported. Cezanne and Trabid share deubiquitinase and zinc finger domains with A20, and Cezanne inhibits TNF-α-induced NF-κB activation (36). ZNF216 is another A20-like protein that has zinc finger domain and inhibits TNF-α-, IL-1β-, and TLR4-induced NF-κB activation via interaction with NEMO (37). It would be interesting to examine whether these A20-like molecules also regulate IRF-3 activation.

Acknowledgments

We thank Drs. D. Wallach, K. Fitzgerald, S. Memet, A. Israël, and T. Taniguchi for providing invaluable materials.

References

1. Sen, G. C. 2001. Viruses and interferons. *Annu. Rev. Microbiol. 55:255.*
2. Taniguchi, T., K. Ogasawara, A. Takaoka, and N. Tanaka. 2001. IRF family of transcription factors as regulators of host defense. *Annu. Rev. Immunol. 19:623.*
3. Takeda, K., T. Kaisho, and S. Akira. 2003. Toll-like receptors. *Annu. Rev. Immunol. 21:335.*
4. Servant, M. J., N. Grandvaux, and J. Hiscott. 2002. Multiple signaling pathways leading to the activation of interferon regulatory factor 3. *Biochem. Pharmacol. 64:985.*
5. Mori, M., M. Yoneyama, T. Ito, K. Takahashi, F. Inagaki, and T. Fujita. 2003. Identification of Ser^{386} of interferon regulatory factor 3 as critical targets for inducible phosphorylation that determines activation. *J. Biol. Chem. 279:9698.*
6. Fitzgerald, K. A., S. M. McWhirter, K. L. Faia, D. C. Rowe, E. Latz, D. T. Golenbock, A. J. Coyle, S. M. Liao, and T. Maniatis. 2003. IKKε and TBK1 are essential components of the IRF3 signaling pathway. *Nat. Immunol. 4:491.*
7. Sharma, S., B. R. tenOever, N. Grandvaux, G. P. Zhou, R. Lin, and J. Hiscott. 2003. Triggering the interferon antiviral response through an IKK-related pathway. *Science 300:1148.*
8. Takeda, K., and S. Akira. 2004. TLR signaling pathways. *Semin. Immunol. 16:3.*
9. Yamamoto, M., S. Sato, H. Hemmi, S. Uematsu, K. Hoshino, T. Kaisho, O. Takeuchi, K. Takeda, and S. Akira. 2003. TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nat. Immunol. 4:1144.*
10. Fitzgerald, K. A., D. C. Rowe, B. J. Barnes, D. R. Caffrey, A. Visintin, E. Latz, B. Monks, P. M. Pitha, and D. T. Golenbock. 2003. LPS-TLR4 signaling to IRF-3/7 and NF-κB involves the Toll adapters TRAM and TRIF. *J. Exp. Med. 198:1043.*
11. Oshiumi, H., M. Sasai, K. Shida, T. Fujita, M. Matsumoto, and T. Seya. 2003. TIR-containing adapter molecule (TICAM)-2, a bridging adapter recruiting to Toll-like receptor 4 TICAM-1 that induces interferon-β. *J. Biol. Chem. 278:49751.*
12. Sato, S., M. Sugiyama, M. Yamamoto, Y. Watanabe, T. Kawai, K. Takeda, and S. Akira. 2003. Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-κB and IFN-regulatory factor-3, in the Toll-like receptor signaling. *J. Immunol. 171:4304.*
13. Beyaert, R., K. Heyninck, and S. Van Huffel. 2000. A20 and A20-binding proteins as cellular inhibitors of nuclear factor-κB-dependent gene expression and apoptosis. *Biochem. Pharmacol. 60:1143.*
14. Buwitt, U., C. Koch, D. Tatje, J. Hoppe, and G. Gross. 1992. Platelet-derived growth factor isoforms AA, AB, and BB differentially activate poly r(I):r(C)-induced genes in human fibroblast FS4 cells. *DNA Cell Biol. 11:641.*
15. Donze, O., J. Deng, J. Curran, R. Sladek, D. Picard, and N. Sonenberg. 2004. The protein kinase PKR: a molecular clock that sequentially activates survival and death programs. *EMBO J. 23:564.*
16. Evans, P. C., H. Ovaa, M. Hamon, P. J. Kilshaw, S. Hamm, S. Bauer, H. L. Ploegh, and T. S. Smith. 2004. A20, a regulator of inflammation and cell survival, has deubiquitinating activity. *Biochem. J. 378:727.*
17. Lee, E. G., D. L. Boone, S. Chai, S. L. Libby, M. Chien, J. P. Lodolce, and A. Ma. 2000. Failure to regulate TNF-induced NF-κB and cell death responses in A20-deficient mice. *Science 289:2350.*
18. O'Reilly, S. M., and P. N. Moynagh. 2003. Regulation of Toll-like receptor 4 signalling by A20 zinc finger protein. *Biochem. Biophys. Res. Commun. 303:586.*
19. Yoneyama, M., W. Suhara, Y. Fukuhara, M. Fukuda, E. Nishida, and T. Fujita. 1998. Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. *EMBO J. 17:1087.*
20. Tojima, Y., A. Fujimoto, M. Delhase, Y. Chen, S. Hatakeyama, K. Nakayama, Y. Kaneko, Y. Nimura, N. Motoyama, K. Ikeda, et al. 2000. NAK is an IκB kinase-activating kinase. *Nature 404:778.*
21. Miyagishi, M., and K. Taira. 2002. U6 promoter-driven siRNAs with four uridine 3′ overhangs efficiently suppress targeted gene expression in mammalian cells. *Nat. Biotechnol. 20:497.*
22. Yamamoto, M., S. Sato, K. Mori, K. Hoshino, O. Takeuchi, K. Takeda, and S. Akira. 2002. A novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-β promoter in the Toll-like receptor signaling. *J. Immunol. 169:6668.*
23. Saitoh, T., H. Nakano, N. Yamamoto, and S. Yamaoka. 2002. Lymphotoxin-β receptor mediates NEMO-independent NF-κB activation. *FEBS Lett. 532:45.*
24. Zhang, S. Q., A. Kovalenko, G. Cantarella, and D. Wallach. 2000. Recruitment of the IKK signalosome to the p55 TNF receptor: RIP and A20 bind to NEMO (IKKγ) upon receptor stimulation. *Immunity 12:301.*
25. Nakagawa, R., T. Naka, H. Tsutsui, M. Fujimoto, A. Kimura, T. Abe, E. Seki, S. Sato, O. Takeuchi, K. Takeda, et al. 2002. SOCS-1 participates in negative regulation of LPS responses. *Immunity 17:677.*
26. Kinjyo, I., T. Hanada, K. Inagaki-Ohara, H. Mori, D. Aki, M. Ohishi, H. Yoshida, M. Kubo, and A. Yoshimura. 2002. SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. *Immunity 17:583.*
27. Kobayashi, K., L. D. Hernandez, J. E. Galan, C. A. Janeway, Jr., R. Medzhitov, and R. A. Flavell. 2002. IRAK-M is a negative regulator of Toll-like receptor signaling. *Cell 110:191.*
28. Brint, E. K., D. Xu, H. Liu, A. Dunne, A. N. McKenzie, L. A. O'Neill, and F. Y. Liew. 2004. ST2 is an inhibitor of interleukin 1 receptor and Toll-like receptor 4 signaling and maintains endotoxin tolerance. *Nat. Immunol. 5:373.*
29. Sakaguchi, S., H. Negishi, M. Asagiri, C. Nakajima, T. Mizutani, A. Takaoka, K. Honda, and T. Taniguchi. 2003. Essential role of IRF-3 in lipopolysaccharide-induced interferon-β gene expression and endotoxin shock. *Biochem. Biophys. Res. Commun. 306:860.*
30. Heylbroeck, C., S. Balachandran, M. J. Servant, C. DeLuca, G. N. Barber, R. Lin, and J. Hiscott. 2000. The IRF-3 transcription factor mediates Sendai virus-induced apoptosis. *J. Virol. 74:3781.*
31. Senger, K., M. Merika, T. Agalioti, J. Yie, C. R. Escalante, G. Chen, A. K. Aggarwal, and D. Thanos. 2000. Gene repression by coactivator repulsion. *Mol. Cell 6:931.*
32. Juang, Y. T., W. C. Au, W. Lowther, J. Hiscott, and P. M. Pitha. 1999. Lipopolysaccharide inhibits virus-mediated induction of interferon genes by disruption of nuclear transport of interferon regulatory factors 3 and 7. *J. Biol. Chem. 274:18060.*
33. Heyninck, K., D. De Valck, W. Vanden Berghe, W. Van Criekinge, R. Contreras, W. Fiers, G. Haegeman, and R. Beyaert. 1999. The zinc finger protein A20 inhibits TNF-induced NF-κB-dependent gene expression by interfering with an RIP- or TRAF2-mediated transactivation signal and directly binds to a novel NF-κB-inhibiting protein ABIN. *J. Cell Biol. 145:1471.*
34. Meylan, E., K. Burns, K. Hofmann, V. Blancheteau, F. Martinon, M. Kelliher, and J. Tschopp. 2004. RIP1 is an essential mediator of Toll-like receptor 3-induced NF-κB activation. *Nat. Immunol. 5:503.*
35. Klinkenberg, M., S. Van Huffel, K. Heyninck, and R. Beyaert. 2001. Functional redundancy of the zinc fingers of A20 for inhibition of NF-κB activation and protein-protein interactions. *FEBS Lett. 498:93.*
36. Evans, P. C., E. R. Taylor, J. Coadwell, K. Heyninck, R. Beyaert, and P. J. Kilshaw. 2001. Isolation and characterization of two novel A20-like proteins. *Biochem. J. 357:617.*
37. Huang, J., L. Teng, L. Li, T. Liu, L. Li, D. Chen, L. G. Xu, Z. Zhai, and H. B. Shu. 2004. ZNF216 is an A20-like and IκB kinase γ-interacting inhibitor of NFκB activation. *J. Biol. Chem. 279:16847.*

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 27, Issue of July 8, pp. 25687–25696, 2005
Printed in U.S.A.

Analysis of Double-stranded RNA-induced Apoptosis Pathways Using Interferon-response Noninducible Small Interfering RNA Expression Vector Library*[S]

Received for publication, November 11, 2004, and in revised form, April 13, 2005
Published, JBC Papers in Press, April 21, 2005, DOI 10.1074/jbc.M412784200

Sahohime Matsumoto‡§¶, Makoto Miyagishi¶‖, Hideo Akashi‡¶, Ryozo Nagai§, and Kazunari Taira‡¶‡‡**

*From the ‡Department of Chemistry and Biotechnology, School of Engineering, the University of Tokyo, Hongo, Tokyo 113-8656, the §Department of Cardiovascular Medicine, Graduate School of Medicine, the University of Tokyo, Hongo, Tokyo 113-8655, ¶Gene Function Research Center, National Institute of Advanced Industrial Science and Technology, Central 4, 1-1-1 Higashi, Tsukuba Science City 305-8562, and **21st Century Center of Excellence Program, Graduate School of Medicine, the University of Tokyo, Hongo, Tokyo 113-8655, Japan*

We have developed an original vector library that allowed us to exploit the phenomenon of RNA interference but also allowed us to avoid the confounding effects of the interferon response. In the present work, we used our library of small interfering RNA expression vectors to examine the genes involved in apoptosis that was induced by double-stranded RNA. To our surprise, screening of our library revealed two novel double-stranded RNA-induced apoptotic pathways, a JNK/SAPK-mediated mitochondrial pathway and an ERK2-related pathway, both of which appeared to be independent of the serine-threonine protein kinase-dependent caspase pathway. We also found that MST2 and protein kinase Cα both activated the pro-apoptotic signal mediated by ERK2. The results of our screening analysis suggested the utility of large scale screenings with libraries of small interfering RNA expression vectors.

RNA interference (RNAi),[1] reported originally by Fire *et al.* (1), is a phenomenon whereby small double-stranded RNAs (dsRNAs) induce the degradation of corresponding target mRNAs. When dsRNAs are introduced into cells, they are processed to yield RNAs of 21–23 nucleotides with overhangs of 2 or 3 nucleotides at their 3′ ends. These small interfering RNAs (siRNAs) (2, 3) are integrated into the RNA-induced silencing complex, and the RNA-induced silencing complex binds to the complementary target mRNA with subsequent degradation of this mRNA (3). However, in mammalian cells, dsRNAs also have nonspecific inhibitory effects, which are known collectively as the interferon response. The use of synthetic siRNAs allows cells to escape the interferon response (2), and such siRNAs are used for the induction of RNAi in mammalian cells. However, synthetic siRNAs are not stable over the long term in cells, and their synthesis is also very expensive.

Several groups, including our own, have circumvented these problems by developing systems for vector-based RNAi (4–10). This approach enables the maintenance of RNAi activity for much longer periods than can be achieved with synthesized siRNAs, and thus, siRNA expression vectors increase the possibility of the application of RNAi as a practical approach to gene silencing.

There are two types of siRNA expression vector, the tandem type and the hairpin type. The hairpin type (11) has sense and antisense nucleotides in a single chain, connected via an loop sequence, whereas the tandem type (12) has separate sense and antisense sequences. Hairpin-type vectors allow rapid formation of stable hairpin structures with strong suppressive activity. By using a hairpin-type siRNA expression vector, we succeeded in constructing a vector system that was genetically stable and had strong suppressive activity (12).

The induction of apoptosis by dsRNA is remarkable, not only with respect to the interferon response that is associated with RNAi but also as a mechanism by which mammalian cells protect themselves against viral infection. However, the relevant mechanisms are poorly understood. It has been demonstrated that interferon (IFN) and dsRNA induce programmed cell death or apoptosis in mammalian cells (13). When dsRNA or a virus is introduced into a cell, the host cell induces the synthesis of IFN as a defense mechanism. IFN is a type of cytokine that induces the expression of a number of intracellular genes for the prevention of viral invasion and facilitates the apoptosis of infected cells. IFN induces expression of a dsRNA-dependent serine-threonine protein kinase (PKR) (14), which regulates protein synthesis via phosphorylation of the α-subunit of eukaryotic initiation factor 2α (15), with resultant apoptosis. PKR also functions as a signal transducer to mediate the activities of transcription factors, such as nuclear factor κB (NF-κB) (16). PKR might also regulate the activities of apoptosis-related proteins, such as p53 (17) and Fas (18, 19), and it plays a role in the activation of the caspase pathway (20). However, full details of the dsRNA-induced apoptotic network remain to be defined.

* This work was supported by various grants from the National Institute of Advanced Industrial Science and Technology (AIST), the New Energy and Industrial Technology Development Organization (NEDO), and the Japan Foundation for Aging and Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains a table.

‖ To whom correspondence may be addressed. Tel.: 81-29-861-3015; Fax: 81-3-5841-7340; E-mail: makoto-m@chembio.t.u-tokyo.ac.jp.

‡‡ To whom correspondence may be addressed. Tel.: 81-3-5841-8828; Fax: 81-3-5841-8828; E-mail: taira@chembio.t.u-tokyo.ac.jp.

[1] The abbreviations used are: RNAi, RNA interference; dsRNA, double-stranded RNA; PKCα, protein kinase Cα; siRNAs, small interfering RNAs; JNK, c-Jun NH_2-terminal kinase; SAPK, stress-activated protein kinase; ERK, extracellular signal-regulated kinase; PKR, serine-threonine protein kinase; IFN, interferon; TUNEL, terminal deoxynucleotidyltransferase dUTP nick-end labeling; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; HCV, hepatitis C virus; MAPK, mitogen-activated protein kinase; VDAC, voltage-dependent anion-selective channel protein.

In the present study, we attempted to identify components of the dsRNA-induced apoptotic pathway by using our library of siRNA expression vectors. We screened ~700 vectors that were targeted against 241 genes, which included genes for apoptosis-related proteins, kinases, transcription factors, and other proteins, and we identified several interesting genes, for example, genes for proteins in the Bcl-2 family and the mitogen-activated protein kinase (MAPK) superfamily (*JNK/SAPK, ERK1/2,* and p38 *MAPK*). We show here that the dsRNA-induced apoptotic pathway is composed of at least three independent pathways as follows: the "classical" PKR-dependent pathway; a JNK/SAPK-mediated mitochondrial pathway; and an ERK2-related pathway. Contrary to a previously accepted hypothesis, ERK2 promoted dsRNA-induced apoptosis.

EXPERIMENTAL PROCEDURES

Construction of siRNA Expression Plasmids—The construction of siRNA expression plasmids was based on the pcPUR hU6 vector (iGENE Therapeutics, Inc.; www.iGENE-therapeutics.co.jp). The vector includes a human U6 promoter, a puromycin resistance gene, and BspMI cloning sites. Because hairpin-type siRNA expression plasmids have higher suppressive activity than tandem-type plasmids, we purchased synthetic oligonucleotides (Takara, Kyoto, Japan) in which sense and antisense nucleotides were connected by an 11-base hairpin loop and formed as a single chain. After annealing, DNA fragments were ligated into the BspMI sites of pcPUR hU6.

Prediction of Target Sites—We constructed our own algorithm for the prediction of target sites of RNAi. This algorithm is based on the partial least squares method (12). We used the algorithm to select at least two target sites in each gene of interest.

Culture and Transfection of Cells and Determination of Cell Viability—We cultured HeLa S3 cells in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum and 1% antibiotics. Transfections were carried out using Lipofectamine™ 2000 (Invitrogen), as described in the manufacturer's protocol. Thirty six hours after transfection, cells were selected by exposure to 1 μg/ml puromycin for 24 h. The pcPUR hU6 vector targeted against the *Renilla* gene for luciferase and hepatitis C virus (HCV) served as a negative control. After selection, individual populations of cells were resuspended in fresh medium and divided among three wells in a 48-well plate, with 1×10^5 cells per well. After 18 h, 0.5 μg of poly(I-C) (Amersham Biosciences) was introduced into cells in the presence of FuGENE™ 6 (Roche Diagnostics). Twenty four hours after transfection with poly(I-C), cells were washed three times with PBS and fixed in 4% paraformaldehyde for 15 min at room temperature prior to staining with 0.2% crystal violet.

Reporter Genes and Assays of Activity—HeLa S3 cells were transfected with 25 ng of firefly luciferase expression vector (pGL3; Promega, Madison, WI) plus 2.5 ng of *Renilla* luciferase expression vector and 0.1, 1, 10, or 100 ng of 50-bp synthesized dsRNA or 0.1, 1, or 10 ng of U6 promoter-driven hairpin vector that encoded a 50-bp hairpin RNA and the control vector (pU6 and pUC 19), in individual wells of 48-well plates. Luciferase activity was analyzed 24 h after transfection using the Dual Luciferase System (Promega). We added empty vectors to ensure that equal amounts of DNA were used in each transfection.

Preparation of Duplexes of RNA Oligonucleotides—By using T7 RNA polymerase (AmpliScribe; Epicenter Technologies, Madison, WI), we prepared sense and antisense RNAs of 50 bp that were targeted against firefly luciferase. The oligonucleotides were purified on a 6% polyacrylamide gel that contained 7 M urea, with subsequent purification by column chromatography and ethanol precipitation. The purified sense and antisense RNAs were annealed to generate duplexes.

TUNEL Assay—Terminal deoxynucleotidyltransferase dUTP nick end-labeling (TUNEL) analysis was performed with an *in situ* Cell Death Detection kit (Roche Diagnostics). Cells on a slide were washed twice with ice-cold PBS and fixed in 4% paraformaldehyde for 15 min at room temperature. After removal by washing with paraformaldehyde, 0.1% Triton X-100 and 0.1% sodium acetate were dropped on the slide, which was then incubated for 2 min on ice. After the slide had been washed again with PBS, 50 μl of TUNEL reaction mix from the kit were placed on the cells, and they were incubated for 60 min at 37 °C. The cells on the slide were then examined under a fluorescence microscope.

Western Blotting Analysis—Cells were collected and washed with ice-cold PBS, lysed in a solution of 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 1 mM Na_2EDTA, 10 mM NaF, 1 mM Na_3VO_4, and Complete Protease Inhibitor mixture (Roche Diagnostics). After sonication, the whole-cell extract was centrifuged at 15,000 rpm for 15 min. The concentration of protein in the supernatant was quantified with a protein assay kit (Bio-Rad) with bovine serum albumin as a standard. Fifteen to 20 μg of protein per lysate were fractionated by SDS-PAGE. Bands of proteins were transferred to an Immobilon™-P^{SQ} membrane (Millipore Corp., Bedford, MA), and the membrane was subjected to immunoblotting with appropriate antibodies. The PKR-specific antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The actin-specific antibody was from Sigma. The antibodies against caspase 3, caspase 7, caspase 10, and Apaf-1 antibodies were obtained from BD Biosciences. Antibodies against caspase 9, BAX, Bid, MST2, PKCα, ERK1/2, phospho-ERK1/2, JNK/SAPK, phospho-JNK/SAPK, p38 MAPK, phospho-p38 MAPK, and caspase 8 were obtained from Cell Signaling Technology (Beverly, MA). Antibodies against VDAC were purchased from Oncogene (Cambridge, MA).

Quantitation of the Release of Cytochrome c from Mitochondria—HeLa S3 cells were washed with ice-cold PBS, resuspended in 100 μl of cytoplasmic buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.4% Nonidet P-40 and Complete Protease Inhibitor mixture), and incubated on ice for 30 min. After homogenization by passage through a needle (25 gauge 5/8-inch), samples were centrifuged at $750 \times g$ for 10 min at 4 °C to pellet nuclei. The resultant supernatant was centrifuged at $20,000 \times g$ for 30 min to generate the cytoplasmic fraction. After quantification of protein as described above, 15 μg of the fractionated proteins were assayed by Western blotting with cytochrome *c*-specific antibody (BD Biosciences).

Rescue Experiment of the siRNA Expression Vector Targeted against ERK2—The construction of ERK2 expression plasmid was generated by subcloning based on RT-PCR into pcEF9, which had an EF1α promoter and a hygromycin resistance gene. The *ERK2* mutation was made by PCR-based site-directed mutagenesis. Three nucleotide mutations were introduced at the target sequence of siRNA expression vector without conversion of the amino acids (G468C, C471G, and C477G), and the resultant plasmid was named pcEF9-ERK2-Rescue. To generate stable cell lines, cells were transfected with 8 μg of the linearized pcEF9 or linearized pcEF9-ERK2-Rescue vectors using Lipofectamine™ 2000 (Invitrogen), as described in the manufacturer's protocol. Thirty six hours after transfection, the cells were selected by 100 μg/ml hygromycin for 2 days, followed by changes with fresh medium with 50 μg/ml hygromycin several times for 3 weeks. For the rescue experiment, the two types of stable cells were transfected with siRNA expression vectors targeted against *Renilla* luciferase (negative control) or ERK2, respectively. Thirty six hours after transfection, cells were selected by 1 μg/ml puromycin for 24 h. After selection, individual populations of cells were resuspended in fresh medium and divided among three wells in a 48-well plate, with 1×10^5 cells per well. After 18 h, 0.5 μg of poly(I-C) (Amersham Biosciences) was introduced into cells as described above.

RESULTS

Use of the siRNA Expression Vector Library Reduces the Interferon Response—One of the most significant advantages of the siRNA expression library, as compared with synthetic siRNA libraries, is the reduction in the interferon response in transfected cells.[2] Although it had been postulated that the interferon response occurs only in response to long dsRNAs and not to siRNAs, it was demonstrated recently that some short siRNAs could also induce the interferon response (21). Therefore, we examined the interferon response induced by various types of short dsRNA in detail. Fig. 1, *A* and *B,* shows a comparison, in terms of the interferon response, between synthetic and vector-derived siRNAs.

In the experiments shown in Fig. 1, we used 50-bp dsRNA against firefly luciferase to sensitize cells. As shown in Fig. 1*A*, the synthesized (*i.e. in vitro* transcribed) short dsRNA, targeted to the firefly gene for luciferase, apparently repressed the expression of the firefly gene in a dose-dependent manner, but the expression of *Renilla* luciferase was also suppressed, suggesting that a nonspecific effect might have occurred because of the interferon response. By contrast, the same short dsRNA

[2] H. Akashi, M. Miyagishi, T. Yokota, T. Watanabe, T. Hino, K. Nishina, M. Kohara, and K. Taira, unpublished data.

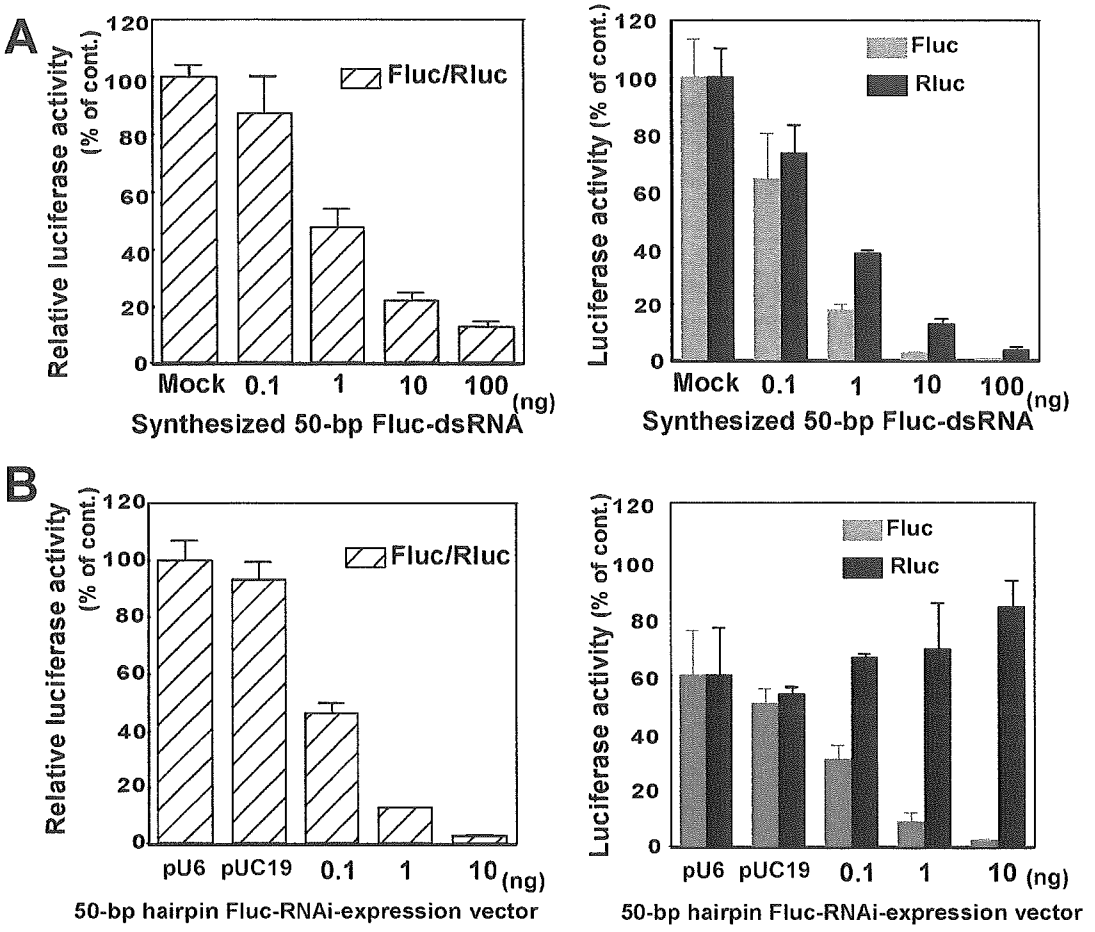


FIG. 1. *A* and *B*, nonspecific inhibition by synthesized (*in vitro* transcribed) 50-bp dsRNA and U6 promoter-driven hairpin RNA targeted against firefly luciferase. *A*, HeLa S3 cells were transfected with 25 ng of firefly luciferase expression vector, 2.5 ng of *Renilla* luciferase expression vector, and the indicated amounts of synthesized dsRNA. The *left panel* shows the relative suppression of firefly luciferase (*Fluc*), standardized by reference to the activity of *Renilla* luciferase (*Rluc*). The *right panel* shows the absolute activities of the firefly and *Renilla* luciferases. *B*, HeLa S3 cells were transfected with 25 ng of firefly luciferase expression vector, 2.5 ng of *Renilla* luciferase expression vector, and the indicated amounts of the 50-bp hairpin RNA expression vector. The *left panel* and the *right panel* show the reductions in relative luciferase activity and absolute luciferase activities, respectively. Each experiment was performed in triplicate, and the results are shown as means ± S.E. *C*, results of TUNEL assays. The two images on the *left* show cells transfected with dsRNA (poly(I-C)). The *yellow arrowheads* indicate apoptotic cells. The *right* pair of images shows untreated controls. *D*, HeLa S3 cells were transfected with 0.5 μg of poly(I-C) as indicated. PARP and cleaved PARP in cell extracts were detected by Western blotting. Western blotting with actin-specific antibodies was performed to confirm that equal amounts of cell extracts had been loaded on the gel.

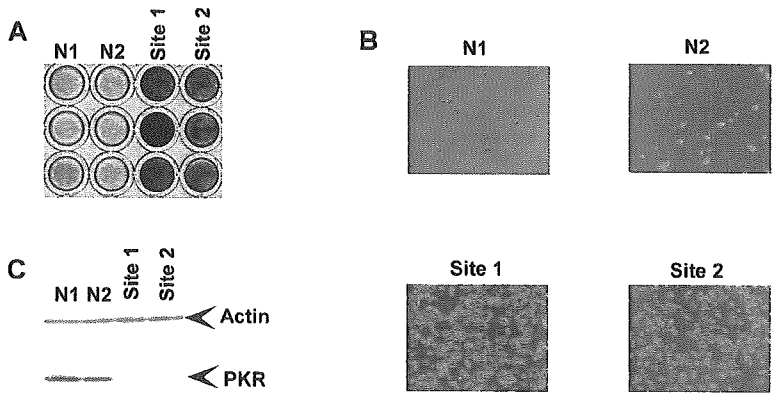


FIG. 2. **Inhibition of dsRNA-induced apoptosis by siRNA expression vectors directed against the gene for PKR.** HeLa S3 cells were transfected with siRNA expression vectors directed against the gene for PKR at two different sites (*Site 1* and *Site 2*) or with negative control vectors (*N1* and *N2*). After selection with puromycin, the cells were transfected with poly(I-C). *A*, plates after staining with crystal violet. *B*, microscopic images of cells. *C*, Western blotting analysis of extracts of cells transfected with siRNA expression vectors directed against the gene for PKR (*Site 1* and *Site 2*) and negative controls (*N1* and *N2*).

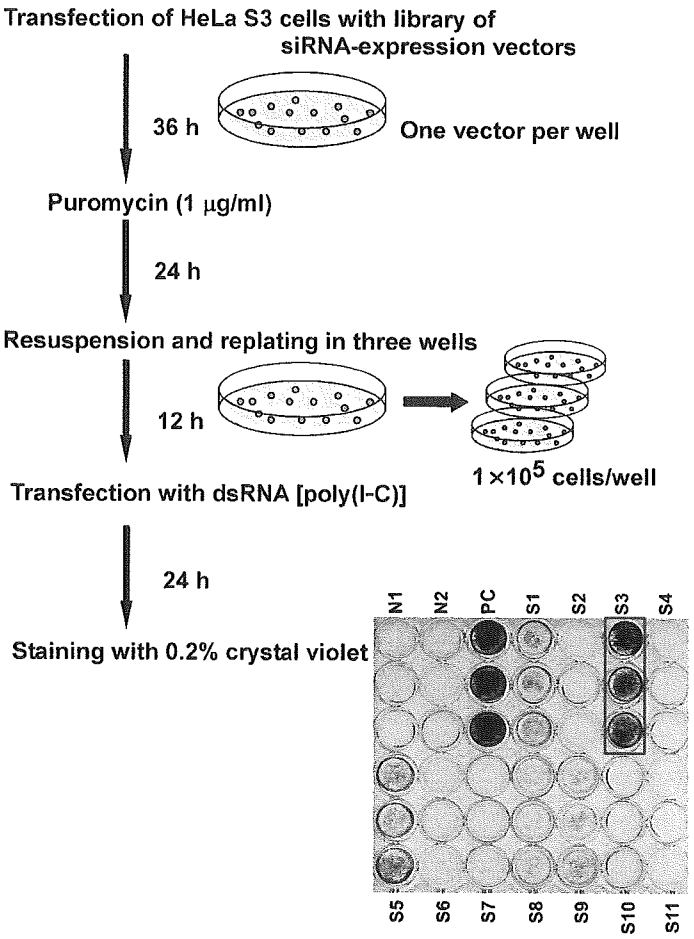


FIG. 3. **Schematic representation of the strategy for screening for pro-apoptotic genes using the library of siRNA expression vectors.** The photograph of wells shows an example of screening for anti-apoptotic genes. *PC*, positive control, siRNA expression vector targeted against PKR was selected as a positive control; *N1*, negative control 1, siRNA expression vector targeted against *Renilla* luciferase; *N2*, negative control 2, siRNA expression vector targeted against HCV; *S1–S11*, sample S1–11, transfected with individual siRNA expression vectors. The wells surrounded by the *red rectangle* contain cells that survived exposure to dsRNA and were resistant to dsRNA-induced apoptosis.

transcribed from a U6 promoter (see below for details) inside cells did not induce the nonspecific reduction in the level of *Renilla* luciferase via the interferon response (Fig. 1*B*). Furthermore, we found that introduction of mutations (either C to U or A to G) in the stem region significantly reduced the interferon response that was induced by long and short dsRNAs.[2] The extent of induction of the response depended on the sequence of the short siRNA, but it appeared to be advantageous to avoid any possibility of a strong interferon response by use of siRNA expression vectors, rather than synthetic siRNAs, for the construction of libraries. Moreover, this feature was especially important, for example, in attempts to analyze the genes involved in dsRNA-induced apoptosis, which includes the PKR pathway.

Optimization of Conditions for Use of the Library of siRNA Expression Vectors—A useful siRNA expression library should have strong suppressive activity and high genetic stability. Although the hairpin-type siRNA expression vectors had highly suppressive activity, 20–40% of our constructs acquired mutations within the hairpin region when such vectors were introduced into *Escherichia coli*. We resolved these problems by designing vectors with several C to T (or A to G) point mutations in the sense region. Such vectors could be sequenced without any problems; they did not exhibit reduced suppressive