青 ペ ー ジ

"イレッサ（ゲフィチニブ）"と"サリドマイド"と
基 礎 医 学 へ の 期 待

桑 野 信 彦

　新しい概念のもとに開発された最近，注目される薬剤に "分子標的薬剤"（Molecular Targeting Drug）がある。がんの代表的増殖因子である上皮増殖因子(EGF)の受容体を標的としたイレッサや，EGF受容体のファミリーの一員であるHER2を標的としたハーセプチンや白血病の発症に関わっているがん遺伝子Bcr-Ablを標的としたグリーベック等がそうである。細胞増殖やDNA複製やRNA合成等を阻害する従来の抗がん剤であるエンドキサンや5-フルオロウラシル，シスプラチン，ドキソルビシン，カンプトテシンとは薬剤開発の発想から臨床効果の評価等が異なることから多くの論議がわが国でもなされている。

　アストラゼネカ社のイレッサが世界に先駆けて最初にわが国で臨床応用が開始されたのは，福岡正博先生をはじめとした臨床腫瘍学者の努力による所が大きい。これに先立ち，カンプトテシンについて日本発の臨床試験研究が権威あるJournal of Clinical Oncologyに発表され日本の臨床研究が国際的信頼を得たことは，有吉寛先生，西條長宏先生や福岡先生をはじめとする，わが国の臨床腫瘍学者たちの科学的根拠に基づく臨床試験の基盤づくりがあったからだということに異論をはさむヒトはいないだろう。

　わが国としては，異例と思えるスピードで2003年にイレッサの臨床応用が開始されるとまもなく，その非小細胞肺癌（NSCLC）への効果に驚嘆の声があがった。と同時に投与患者の数％に生じる間質性肺炎のリスクをメディアは大々的に報告し

た。多くの治療薬の投与で経験される間質性肺炎が，イレッサに特徴的な副作用であるかのような社会的風潮を根づかせはじめた。その頃，イレッサのNSCLCの臨床効果について，①男性より女性に有効性が高い。②腺癌が上皮性癌より有効性が高い。③日本人が欧米人に較べ有効性が高い。等 "イレッサの不思議" として注目されていた。この疑問に答えて2004年に米国の2つの研究グループから独立に報告があった。癌にEGF受容体のチロシンキナーゼ・ドメインに突然変異が見出された肺癌患者はイレッサに応答性が高いこと，さらに日本人女性や腺癌にこの突然変異の頻度が高いこと等に関する報告であった（Lynch et al., New Engl. J. Med., 350: 2129, 2004; Paez et al., Science, 304: 1497, 2004）。一方，これらの発表と同じ頃，わが国からも肺癌細胞の細胞死や増殖がどれだけEGF受容体のシグナルに依存しているかが，イレッサ感受性の重要な鍵であることが報告された（Ono et al., Molec. Cancer Therapeut., 3: 465, 2004）。このEGF受容体シグナルの肺癌細胞の細胞死/増殖への緊密な依存度は，突然変異の有無に影響されるというのが結論である。"肺癌患者に効果がみられる薬が登場したことを肺癌の臨床医としてはじめて経験することができました。" と愛知県がんセンターの光冨徹哉博士が語っていたのが印象的であった。このイレッサをはじめとしてその分子標的の存在や発現様式を把握しながらがん治療を進めることは，いわゆる科学的根拠（Principle of Proof）に基づいたオーダーメイド医療の実践でもある。

久留米大学・先端癌治療研究センター　所長

他方，妊婦をはじめとして鎮痛また催眠薬としてその当時の化学者たちが最高の発明だと自信を持って世へ送り出したのが合成薬サリドマイド (Thalidomide) であった。半世紀前のサリドマイド投与による畸型児サリドマイドベービーは，人類に対してくすりの恐ろしさを示す，繰り返してはならない薬害の大きな警告であった。唯この50年間サリドマイドの催畸作用だけでなく生物活性と構造に関する研究が続けられてきたのは，非常な驚きである。血管新生を阻害したというFolkman博士の研究室からの報告も再現性については問題があるけれども多大な注目をあつめた。サリドマイドの多発性骨髄腫に対する臨床応用が進められている(唯，妊婦を除くという条件付で)。しかし，このサリドマイドについて最も難しいと考えられる点はその抗腫瘍効果の作用機序がはっきりしていないことである。我々の研究室でもサリドマイドの仕事を続けてきたけれども，ことごとく失敗を繰り返して1報の報告もできなかった。

標的がはっきりしているイレッサとはっきりしていないサリドマイドの2つをとりあげた。"薬を投与する際に毎日を忙しく頑張っている医師たちが薬の作用のしくみや患者の適応性について充分考慮する十分な時間があるのだろうか？" また"投与する薬のことだけでなく，目の前の患者に関する科学的根拠に基づいた病態を把握できているのだろうか？" などと疑問がわいてくる。そんな時に，私は基礎医学の重要性を考えてしまうのである。学生が入学して最初の教養課程（今はこんなことばはないかも知れないが）での生物学をはじめとして，科学の魅力を，より魅力的に語りかける基礎医学教育が極めて大切ではなかろうかと思ってしまうのである。私が専攻した生化学や医化学をはじめとして解剖学，生理学，公衆衛生学や薬理学などの基礎医学を学生諸君に臨床医学以上の魅力で語りかけることが大切である。健康や病気に関する科学の基盤を徹底的に学び，医学の根幹をなす基礎医学を真剣に学ぶことこそイレッサやサリドマイドなどの新薬をベッドサイドで使用していく上で極めて重要である。

病気を科学し，その診断や治療を実践していくために基礎医学と臨床医学は対等のパートナーであるべきである。対等のパートナーとなるためには，基礎医学は医学教育の実践に今までの殻を破って本当の全力投球をやる時代がきている。イレッサもサリドマイドもその作用に関する科学を学ばずして患者への投与はあるべきではない。十分な時間を費やして学生と教授をはじめとする教官は顔と顔をあわせて，一緒に医学を学ぶ姿を目指していくべきであろう。どんな秀才も知識だけではコンピューターに負ける。科学的根拠のもとに目の前の一つ一つの病気の診断と治療を進めることができるための教育と研究の環境づくりと医学の担い手を育成することは，緊急性のある重要課題であろう。アメリカ流の研修医制度の導入は，わが国の臨床医学の混乱だけでなく基礎医学の教育と研究へ深刻な問題を新たに生じさせている。基礎医学をわが国で，わが国にマッチするやり方でどう育成していくかは今我々につきつけられた課題である。私自身もこれまでを反省しながら新たにこの課題にとりくんでいきたいと考えている。

血管新生阻害剤
Angiogenesis-targeting drugs

桑野信彦(写真)　小野眞弓
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◎がんは自己増殖する能力を有しているが，ある一定以上の大きさになるためには血管による栄養の供給を必要とする．したがって，がんでつくられる新生血管を標的とした治療薬は，がんの増大を阻害することが期待される．さらに，血管新生ががんの浸潤や転移と密接に関連していることから，血管新生阻害剤が転移を阻害することも期待されている．最近，代表的な血管新生因子VEGFを標的とした抗体治療薬Avastin®が，抗がん剤との併用で進行性大腸癌をはじめ難治性がんに有効性が報告され，多大の注目を集めている．がんの血管新生のメカニズムやネットワークシステムを基盤にした新しい有効な分子標的薬剤の登場に期待したい．

Key word 血管新生阻害剤，VEGF/VEGFレセプター，がん血管新生，アバスチン®

従来のがん化学療法剤の開発はがん細胞自身の増殖や細胞周期に関連する酵素や因子に加えて，DNA，RNAや蛋白合成などを対象に進められてきた．これと対照的にがんの間質応答のひとつ"新生血管"を標的とした治療薬の開発は，Folkman博士の発表以来，大きな期待が寄せられた(Folkman，1971)．その結果，VEGFをはじめとした血管新生関連因子を標的とした治療薬の開発研究が積極的に進められてきた．すなわち，エンドスタチンやアンジオスタチンをはじめとした内在性血管新生抑制因子，matrix metalloproteinases(MMPs)阻害剤，血小板第IV因子(PF-4)，メチオニンアミノペプチダーゼ(Met AP-2)などのプロテアーゼ阻害剤，フマジリン誘導体，インテグリン阻害剤，またVEGFレセプター(VEGFR)阻害剤などを含む多くの血管新生阻害剤が開発され，臨床応用が開始された[1-3]．しかし最近まで，確実な臨床効果が報告されたものはなかった．現在，臨床試験が進められている血管新生阻害剤を表1に示している．

本稿では，2003年のアメリカ臨床腫瘍学会(ASCO)で臨床効果が報告された抗VEGFヒト型モノクローナル抗体Bevacizumab(Avastin®)やがんの炎症応答と関連する血管新生について述べる．

臨床試験で効果が報告された抗血管新生治療薬——VEGFを標的として

VEGFは血管内皮細胞に特異性の高い分子量34～45kDaの増殖因子で，血管透過性因子(VPF)と同一である．VEGFの発現亢進は多くの固形腫瘍をはじめとして，糖尿病性網膜症や関節リウマチなどの疾患でも報告されている．VEGFはさまざまなサイトカインや増殖因子，低酸素，低グルコースなどによって誘導される．VEGFに対して親和性を示すレセプターとしてVEGFR-1(Flt-1)，VEGFR-2(KDR/Flk-1)，VEGF-3，Neuropilin-1，Neuropilin-2などが知られている．それぞれのVEGFファミリーのリガントに対応して血管内皮とリンパ管内皮で働くレセプター群を表2に示している．血管新生の誘導にもっとも深く関与すると考えられているVEGF-A/VEGFR-2のシグナル機序が明らかになってきた．さらに，近年，リ

表 1 臨床試験が進められている血管新生阻害性作用のある分子標的薬剤

薬剤	薬剤タイプ	分子標的	機序
PTK787	低分子	VEGF-R1/2/3*	チロシンキナーゼ
SU6668	低分子	VEGF-R2 PDGF-Rβ	チロシンキナーゼ
SU11248	低分子	VEGF-R2 PDGF-Rβ	チロシンキナーゼ
AZD6474	低分子	VEGF-R2	チロシンキナーゼ
AZD2171	低分子	VEGF-R3	チロシンキナーゼ
CEP-7055	低分子	VEGF-R1/2/3	チロシンキナーゼ
CP-547.632	低分子	VEGF-R2	チロシンキナーゼ
786034	低分子	VEGF-R2	チロシンキナーゼ
IMC-1C11	抗体	VEGF-R2	拮抗作用
Angiozyme	リボザイム	VEGF-R1	mRAN 切断
Avastin®	抗体	VEGF	拮抗作用
VEGF-Trap	可溶型 VEGF-R	VEGF	拮抗作用
Vitaxin	抗体	$\alpha_V\beta_3$-integrin	拮抗作用
EMD121974	低分子	$\alpha_V\beta_3$-integrin	拮抗作用

*VEGF-R1/2/3：VEGF 受容体 1 型, 2 型, 3 型.

表 2 VEGFファミリーと関連レセプターの血管新生とリンパ管新生への関与

リガント	レセプター	標的内皮細胞	生物活性
VEGF-B PlGF VEGF-A	VEGFR-1 (Flt-1)	血管内皮	血管新生
(VEGF-B) (PlGF) (VEGF-A)	Neuropilin-1	血管内皮	血管新生
VEGF-A VEGF-C VEGF-D	VEGFR-2 (KDR)	血管内皮	血管新生
VEGF-A VEGF-C VEGF-D	VEGFR-2	リンパ管内皮	リンパ管新生
(VEGF-A) (VEGF-C) (VEGF-D)	Neuropilin-2	リンパ管内皮	リンパ管新生
VEGF-C VEGF-D	VEGFR-3	リンパ管内皮	リンパ管新生

（　）で示すリガントは比較的弱い活性.

ンパ管新生を誘導する VEGF-C/VEGFR-3 の役割も注目されている. すなわち, 表 3 に示すように血管とリンパ管に発現しているマーカーがつぎつぎに単離され, 血管新生とは異なるリンパ管新生の分子機序が提示されている. VEGFR-1 や VEGFR-2 また Neuropilin-1 などは血管内皮に比較的特異的に発現しているために, これらを標的

にすることにより選択的な血管新生阻害が期待された. しかし, VEGFR-1 や VEGFR-2 を標的とした低分子阻害剤は動物実験系では非常に抗腫瘍や転移阻害活性を示しても, 臨床試験においてはいまだ確実な効果が観察されなかった. その理由についてははっきりしていない.

他方, アメリカの Genentech 社が VEGF-A を標

表 3 血管とリンパ管に発現している分子マーカー

マーカー	血管	リンパ管	発現細胞と組織
Prox 1	−	＋	眼, CNS, IS, 肝, 膵
LYVE-1	−	＋	マクロファージ, 血管類洞内皮(肝, 膵)
Podoplanin	−	＋	破骨細胞, 肺口胞細胞, 腎ポドサイト, 上皮ケラチノサイト
VEGFR-3	−	＋	がん血管, マクロファージ, 樹状細胞
Neuropilin-2	−	＋	ニューロン細胞
Desmoplakin	−	＋	上皮細胞, 心筋
Laminin	＋	−/(＋)	ECM, 基底ラミナ
CollagenIV	＋	−/(＋)	上皮性基底ラミナ
Neuropilin-1	＋	−	ニューロン細胞
CD34	＋	−	血球前駆細胞
VEGFR-1	＋	−	単球/マクロファージ
CD31(PECAM-1)	＋	(＋)	
VEGFR-2	＋	＋	

CNS：中枢神経系.

表 4 転移性大腸癌患者に対するVEGFモノクローナル抗体Avastin® の5-FU/LV(5-FU＋ロイコボリン)との併用効果

治療レジメ	5-FU/LV	5 mg/kg Avastin® ＋ 5-FU/LV	10 mg/kg Avastin® ＋ 5-FU/LV
患者数	$n=36$	$n=35$	$n=33$
生存期間中央値(月)	13.6	17.7	15.2
病状進行なし生存期間中央値(月)	5.2	9.0	7.2
臨床的奏効率(%)	17%	40%	24%

的にした中和抗体 RHnMAb である Bevacizumab (Avastin®)を開発した. Genentech 社の Ferrara 博士らは多くのヒトがんの xenograft モデル系でがんの増大や転移に VEGF 抗体が有効であることを数多く報告した[4].

Avastin® はまず進行性がんに対しての臨床応用が開始され, 2003 年の ASCO で進行性(転移性)大腸癌に対してイリノテカンや 5-FU/LV との併用で, 生存期間の延長が観察された(表 4)[5]. 表 4 に Avastin® と 5-FU/LV との併用効果を示している. 他方, Avastin® はタキソール系抗がん剤, MMP 阻害剤, トポテカン, ドキソルビシンなどとの併用が xenograft モデル系の乳癌, 前立腺癌, 膵癌, 卵巣癌, 神経芽細胞腫, Wilms 腫瘍などに対して抗腫瘍効果を示すことが数多く報告されている. そのなかでヒト卵巣癌細胞株の xenograft モデルで, パクリタキセルと Avastin® との併用に関する抗腫瘍と腹小貯留に対する効果が報告された(図 1)[6].

2005 年の ASCO においても Avastin® は大きな注目を集めている. 治療抵抗性で進行性の大腸癌に対しても Avastin® 単独では効果はみられないが, 併用化学療法剤の治療効果を Avastin® は確実に上昇させている. Jain らは, VEGFR-2 の抗体で治療するとがんの血管構築を正常血管のそれに近いものにすることより, 抗がん剤のがんへの蓄積が増大する可能性を示唆している[7]. 同様な効果が Avastin® でも期待されている. 現在大腸癌だけでなく多くの他のがんに対しても化学療法剤の併用の臨床試験が進められている.

これから期待したい炎症と関連する血管新生阻害剤──NF-κBとCOX2を標的として

Virchow 博士ががんの病態に炎症が深くかかわっていることを提言して以来長い月日が流れている. がんと炎症の問題がいま, 重要な研究対象として注目を集め, がんと炎症を関連づける具体的なシグナル因子や標的細胞が明らかにされはじ
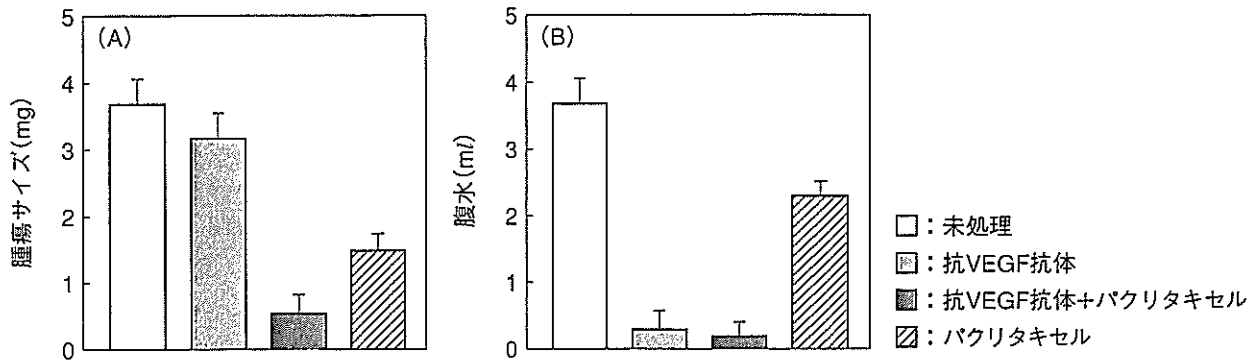
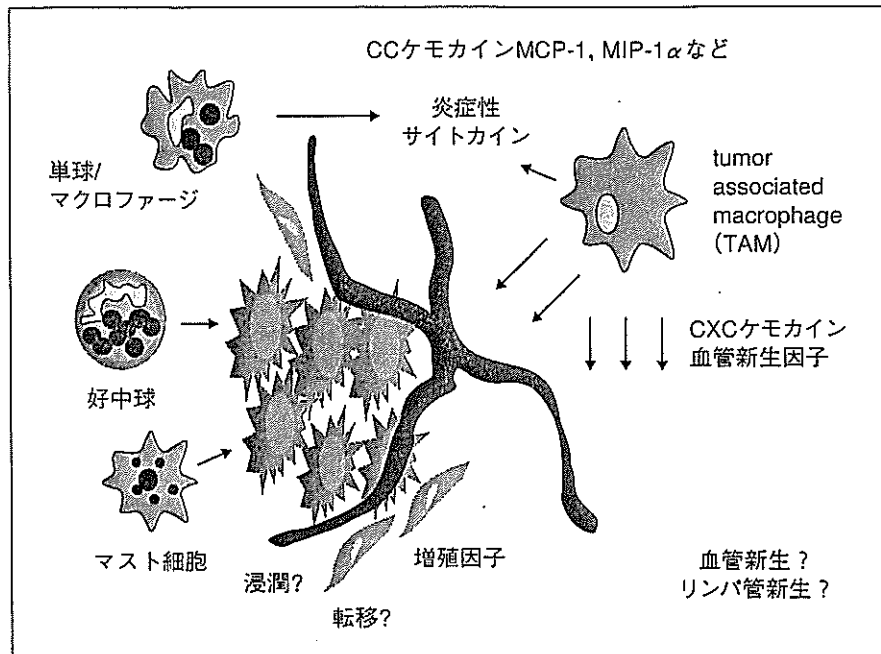図1　ヒト卵巣癌xenograftの腫瘍増殖(A)と腹水貯留(B)に対する抗VEGF抗体とパクリタキセルの作用
平均値±標準誤差，$n=12$.

凡例：
□：未処理
▦：抗VEGF抗体
■：抗VEGF抗体＋パクリタキセル
▨：パクリタキセル

図2　がん間質の炎症応答としてのがん関連マクロファージ(TAM)の関与
TAM はがんの間質の血管新生誘導に深く関与している．TAM の浸潤は，乳癌，子宮頸癌，肺癌，膀胱癌，グリオーマ，メラノーマなどで予後不良と密接に相関を示す．

めている．ヘリコバクターピロリ菌と胃癌や，肝炎 B や C 型ウイルス感染と肝癌との関係など実に多くの癌で，炎症ががん化のメカニズムに関与していることが知られている[8-10]．NF-κB は重要な炎症標的のひとつであり，炎症反応を担って細胞死，発癌や薬剤や放射線耐性に深く関与している．最近 Karin 博士らは，Okayasu 博士(北里大学)らの報告した，塩によって慢性炎症を起こしやがて大腸癌を発症させるモデル系を駆使して，NF-κB のシグナルを阻害することによって大腸癌の発症を著明に減少させることに成功した[11]．さらに他の実験モデル系で，NF-κB を阻害すること

により肝癌の発症を著明に減少させたり，Ras でがん化した乳癌細胞の肺転移を減少させることも報告されている．現在多くの NF-κB を標的とした阻害剤が開発されており，臨床効果のある薬剤の登場に期待したい[12]．

NF-κB の炎症シグナルと関連して乳癌の実験モデル系で，マクロファージが CSF-1 によって遊走蓄積すると乳癌の増大や転移を促進することも報告されている[13]．ジキルとハイドの二面性をもつマクロファージのうちがんの間質に蓄積するものは tumor-associated macrophage(TAM)とよばれ，多くのヒトがんで悪性度と有意に相関することが
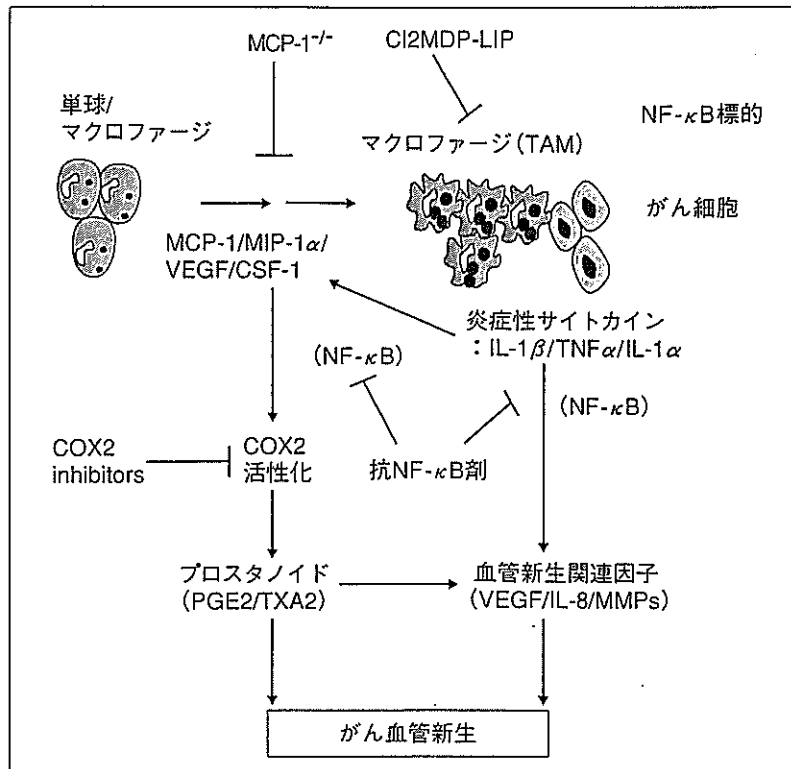
図 3 現在著者らが考えているがんの血管新生や浸潤/転移における
炎症性サイトカイン，NF-κB，COX2とマクロファージの関与

知られている[14]．がんの間質応答として炎症と関連する血管新生が重要な役割を果たしているのではないかと期待されている．図 2 に TAM のがんの間質応答に関与するモデルを提示している．

アラキドン酸カスケードの律速酵素であるシクロオキシゲナーゼ(COX)には，これまでに 2 種類のアイソザイム(COX1, COX2)が同定されている．アスピリンなどに代表される COX 阻害剤がしばしば消炎や鎮痛効果を示すことからもわかるように，COX もまた炎症の代表的な担い手である．COX1 も COX2 も，従来の非ステロイド性抗炎症薬(NSAIDS)であるアスピリンやインドメタシンで阻害される．NSAIDS を長期にわたって服用していたヒトは，大腸癌による死亡率が 40～50％低いことが報告されたことから，がんの予防効果が明らかになった．また，大腸癌を誘導する家族性ポリポーシスのモデル動物では，COX2 の阻害は大腸のポリープ形成を抑制することも報告されている．Tsujii らにより[15]，COX2 強制発現大腸癌細胞では PGE2 や VEGF，bFGF，PDGF などの血管新生促進因子の産生やヒト臍帯由来血管内皮細胞の遊走活性や管腔形成を亢進すること，これらは

COX2 特異的阻害薬で濃度依存性に抑制されることが報告された．

炎症性サイトカインである IL-1β がマウス角膜で著明に血管新生を誘導し COX2 阻害剤がほぼ完全に阻害すること，さらにプロスタノイドのうち PGE2 や TXA2 がとくに強く血管新生を誘導することなどを著者らのグループは報告している[16]．IL-1β で誘導した血管新生に向かって遊走してくる炎症性細胞のなかに多数のマクロファージが観察され，COX2 陽性であることも観察されている[16]．さらに実験モデルマウスにおいて，血中のマクロファージを標的薬剤で除去すると，IL-1β による血管新生が著明に減少することを観察している[17]．図 3 に著者らが現在考えているモデルを示す．がんの間質における炎症性の血管新生を標的にして COX2 や NF-κB やマクロファージなどを標的とした新しい抗血管新生治療戦略を考えていくことが大切と考えている．

おわりに

VEGF に対するモノクローナル抗体の臨床効果のニュースはこの分野で仕事をしてきた者が勇気

づけられるものであった．ただ，VEGF 抗体に効果があって VEGFR-1/VEGFR-2 の阻害剤に明らかな臨床効果が報告されなかったのはたいへん不思議に思われる．"がんの血管新生に VEGF の関与がどれだけかかわっているのであろうか"という疑問とともに，"VEGF 抗体がリンパ管新生には影響しないのであろうか"といった疑問なども生じてくる．がんの悪性形質の獲得に関与する血管新生の全体像を把握して，あらたな分子標的と治療戦略を探索していく努力が大切であろう．

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Characterization of the 5'-untranslated region of YB-1 mRNA and autoregulation of translation by YB-1 protein

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ABSTRACT

The eukaryotic Y-box binding protein YB-1 is involved in various biological processes, including DNA repair, cell proliferation and the regulation of transcription and translation. YB-1 protein is abundant and expressed ubiquitously in human cells, functioning in cell proliferation and transformation. Its concentration is thought to be highly regulated at both the levels of transcription and translation. Therefore, we investigated whether or not the 5'-UTR of YB-1 mRNA affects the translation of YB-1 protein, thus influencing expression levels. Luciferase mRNA ligated to the YB-1 mRNA 5'-UTR was used as a reporter construct. Ligation of the full-length YB-1 5'-UTR (331 bases) enhanced translation as assessed by in vitro and in vivo translation assays. Deletion constructs of the YB-1 5'-UTR also resulted in a higher efficiency of translation, especially in the region mapped to +197 to +331 from the major transcription start site. RNA gel shift assays revealed that the affinity of YB-1 for various 5'-UTR probe sequences was higher for the full-length 5'-UTR than for deleted 5'-UTR sequences. An in vitro translation assay was used to demonstrate that recombinant YB-1 protein inhibited translation of the full-length 5'-UTR of YB-1 mRNA. Thus, our findings provide evidence for the autoregulation of YB-1 mRNA translation via the 5'-UTR.

INTRODUCTION

Y-box proteins function as transcriptional and translational regulators of gene expression. They are found among prokaryotes and eukaryotes and are characterized by the evolutionary conservation of a cold shock domain (CSD). Recently, it was reported that a major protein component of messenger ribonucleoprotein (mRNP) particles in somatic cells is a member of the Y-box binding transcription factor family. This protein acts either as a repressor or an activator of protein synthesis (1–4). It has been hypothesized that YB-1 might play a role in promoting cell proliferation through the transcriptional regulation of various genes, including epidermal growth factor receptor, thymidine kinase, DNA topoisomerase II and DNA polymerase (5,6). The multiple biological roles of YB-1 include the modification of chromatin, the translational masking of mRNA, participation in a redox signaling pathway, RNA chaperoning and regulation of the stress response (7).

It has also been demonstrated that eukaryotic Y-box proteins regulate gene expression at the level of translation by binding directly to RNA (8,9). The rabbit Y-box protein, p50, is found in cytoplasmic mRNP particles in somatic cells and regulates translation by interacting with mRNA (2). The murine MSY1 protein and chicken Y-box protein both regulate transcription and translation (7,10–12). Furthermore, the Y-box family proteins, Xenopus mRNP3/mRNP4 and mouse MSY2, have also been found to be mRNA-masking proteins in germinal cells (13–15). Chen et al. (16) have reported that YB-1 is involved in the mRNA stability of the cdk4 gene; this stability is achieved by the binding of YB-1 to a specific sequence of the mRNA. YB-1 also stabilizes cap-dependent mRNA, since depletion of YB-1 results in accelerated mRNA decay (17).

Previously, we identified several proteins as partners of YB-1, including YB-1 itself, iron regulatory protein 2 (IRP2) and the ribosomal proteins S3A, L18A, L5, L23A and S5. We also provided evidence that YB-1 is involved in the translational regulation of an iron-related protein (18). Y-box binding proteins thus appear to perform critical functions in both

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mRNA turnover and translational control. Considering the important cellular functions of YB-1, it is possible that its expression is highly regulated in eukaryotes. In fact, the YB-1 gene behaves like a primary response gene. Stimulation of mammalian cell proliferation in culture or *in vivo* results in increased YB-1 synthesis. The cellular level of YB-1 is usually controlled by regulating the translation of its mRNA. It is thought that an increase in the cellular YB-1 concentration could alter the translation and stability of some mRNAs. Therefore, several pathways exist to control the function of this important cellular protein.

The 5'- and 3'-untranslated regions (UTRs) of eukaryotic mRNAs are known to play a crucial role in post-transcriptional regulation that modulates nucleo-cytoplasmic mRNA transport, translation efficiency, subcellular localization and stability (19). Several regulatory signals have already been identified within the 5'- or 3'-UTR sequences (20). These signals tend to correspond to short oligonucleotide tracts, able to fold into specific secondary structures which provide binding sites for various regulatory proteins (21–23).

To examine how YB-1 mRNA translation is regulated in eukaryotic cells, we examined the possible role of its relatively long 5'-UTR. Deletion of the YB-1 mRNA 5'-UTR enhances translational activity in both *in vitro* and *in vivo* systems. The affinities of YB-1 for 5'-UTR probe sequences of various lengths were evaluated by RNA gel shift assays; the affinity of YB-1 was higher for the full-length 5'-UTR than for truncated sequences. The addition of recombinant YB-1 inhibited translation through the 5'-UTR of its mRNA; this effect was particularly marked when the full-length 5'-UTR was used. In this study, we have demonstrated for the first time that the 5'-UTR region of human YB-1 mRNA plays an important role in determining the conditions of YB-1 biosynthesis at the translational level.

MATERIALS AND METHODS

Construction of fusion protein expression plasmids

The plasmids containing full-length glutathione *S*-transferase (GST)–YB-1 cDNA fusions, GST–YB-1 deletion mutants and Flag–YB-1 were described previously (24–26).

Reporter gene constructs

A pT7 control plasmid, for *in vitro* transcription and translation experiments, was constructed by digesting luciferase cDNA of a pGL3 basic vector (Promega, Madison, WI) with EcoRI, blunting with Klenow enzyme, and ligation to pT7Blue3 (Novagen, Madison, WI). The pT7-YB5'-1 plasmid was constructed as follows. The entire length of the YB-1 5'-UTR was amplified by PCR from human YB-1 cDNA. The forward primer was 5'-AGGCAGGAACGGTTGTAGGT-3' and the reverse primer was 5'-gtttttggcgtcttccat-GGTTGCGGTGATGG-3'. The latter contains a luciferase coding sequence at the 5'-end (shown in lower case). A luciferase cDNA fragment was also amplified by PCR from a pGL3 basic vector, using the forward primer 5'-CCATCACCGCAACCatggaagacgccaaaaac-3', complementary to the reverse primer of the YB-1 5'-UTR and the reverse primer 5'-ttacacggcgatctttcc-3'. Each PCR-amplified fragment was ligated with the complementary primer regions and

amplified by PCR using the complementary primer pair. The YB-1 5'-UTR-ligated luciferase cDNA fragment was cloned into the EcoRI-digested pT7Blue3 vector in order to generate plasmid pT7-YB5'-1. To functionally characterize the 5'-UTR of the human YB-1 gene, a series of 5'-deletion plasmids (pT7-YB5'-2–pT7-YB5'-6) were amplified by PCR using the pT7-YB5'-1 plasmid as a template. The forward primers were 5'-AAGGTCCAATGAGAATGGAGGA-3' (pT7-YB5'-2), 5'-AAGCTAGGGATTGGGGTCAG-3' (pT7-YB5'-3), 5'-CCT-AGGGCGGGTCGCTCGTA-3' (pT7-YB5'-4), 5'-CGATCG-GTAGCGGGAGCGGAG-3' (pT7-YB5'-5) and 5'-CCG-CCGCCGCCGGCC-3' (pT7-YB5'-6). Each of the PCR-amplified fragments were cloned into the EcoRI-digested pT7Blue3 vector to generate the pT7-YB5'-2–pT7-YB5'-6 plasmids. To construct pCMV and pCMV-YB5'-1–pCMV-YB5'-6 plasmids suitable for expression in mammalian cells, the pT7 or pT7-YB5'-1–pT7-YB5'-6 plasmids were digested with EcoRI. The fragments of luciferase cDNA were ligated into the EcoRI-digested pIRES-EYFP vector (Clontech, Palo Alto, CA), using various sizes of the YB-1 5'-UTR region (pCMV-YB5'-1–pCMV-YB5'-6); a non-ligated fragment (pCMV) was used as a control.

Site-directed mutagenesis of the YB-1 5'-UTR in pT7-YB5'-1 was performed using a PCR-based method. To obtain pT7-YBM1–pT7-YBM3, the full length of the YB-1 5'-UTR sequence was first amplified by PCR. The forward primers were 5'-GGTGGGCAGTACATCAGTACCACTGG-3' (pT7-YBM1), 5'-GCGGGTCGCTAGAGAGGCTTATCCCGC-3' (pT7-YBM2), 5'-CATTCTCGCTAGAACAGTCGGTAG-CGGG-3' (pT7-YBM3), and the reverse primers were 5'-CCAGTGGTACTGATGTACTGCCCACC-3' (pT7-YBM1), 5'GCGGGATAAGCCTCTCTAGCGACCCGC-3' (pT7-YBM2) and 5'-CCCGCTACCGACTGTTCTAGCGAGA-ATG-3' (pT7-YBM3). A second PCR was then performed with Taq polymerase using the first PCR products as templates. The PCR products were cloned into the EcoRI-digested pT7Blue3 vector in order to generate the pT7-YBM1–pT7-YBM3 plasmids. All constructs were confirmed by sequencing using a DNA sequencing system (model 377; Applied Biosystems, Foster City, CA).

Cell lines

A human epidermoid cancer cell line, KB3-1, was cultured in MEM supplemented with 10% newborn calf serum. The human lung cancer cell line H1299 was cultured in RPMI supplemented with 10% fetal bovine serum (FBS). HUVECs were isolated from individual human umbilical cord veins by collagenase digestion and were routinely cultured on type 1 collagen-coated plates in endothelial cell growth medium (Clonetics, Boston, MA) supplemented with 2% FBS. Tissue samples were obtained under an Institutional Review Board approved protocol, after the subjects had provided informed consent. The cells were maintained under standard cell culture conditions at 37°C and 5% CO_2 in a humid environment.

Recombinant proteins and antibodies

Recombinant proteins were expressed in *Escherichia coli* DH5α. YB-1 and YB-1 deletion mutants were purified as GST fusion proteins as described previously (25). Briefly, GST fusion protein expressed in bacteria was induced by incubation with isopropyl-1-thio-β-D-galactopyranoside and cells were

lysed by sonication in 1 ml of binding buffer [1 mM ditiothreitol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 200 mM NaCl, 10% v/v glycerol, 1% Triton-X, in phosphate-buffered saline (PBS) pH 7.3]. Cellular debris was removed by centrifugation and the supernatants were subjected to affinity column chromatography using glutathione–Sepharose 4B (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's recommendations. Antibody to YB-1 was generated as described previously (27).

Primer extension by reverse transcriptase

The primer extension experiments were carried out as described previously (28). Total RNA was prepared from each cell line using an RNeasy Miniprep Kit (Qiagen, Chatsworth, CA) and a QIAshredder microspin homogenizer according to the manufacturer's recommendations. The poly(A)$^+$ RNA was isolated from the total RNA using a Poly(A)$^+$ Isolation Kit from Total RNA (Nippon Gene Co. Ltd, Tokyo, Japan). The primer for the primer extension analysis, 5'-GCTCATGGTTGCGGTGATGG-3', was synthesized to hybridize the sense strand between nucleotides −14 and +6 in the first exon of the YB-1 gene. The synthetic primer was labeled at its 5'-end with [γ-^{32}P]ATP using T4 polynucleotide kinase and hybridized to 1 µg poly(A)$^+$ RNA in 80% formamide, 0.4 M NaCl, 40 mM PIPES (pH 6.4) and 1 mM EDTA for 4 h at 50°C. After precipitation, the nucleic acid pellet was dissolved in reverse transcriptase buffer (Invitrogen Corp., Carlsbad, CA). The primer was extended with 200 U of SuperScript II RNase H$^-$ reverse transcriptase (Invitrogen) using 1 mM each of the four deoxynucleotides. After 1 h at 37°C, the reaction was neutralized and the DNA was collected. The reaction products were analyzed on a 7 M urea–8% polyacrylamide gel to determine the size of the extended product. The gel was exposed to an imaging plate and the blots were visualized and quantified using a phosphorimaging analyzer (model BAS 2000; Fuji Photo Film Co., Tokyo, Japan) and the Image Gauge (version 3.4) program.

RNA immunoprecipitation assay

Cells (100 mm dishes) were transfected with 5 µg of Flag–YB-1 plasmid DNA using LipofectAMINE 2000 reagent (Invitrogen). After 48 h, the cell extract was preincubated with protein A/G–agarose in TNE buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml aprotinin] for 1 h at 4°C with rotation. After centrifugation, the supernatant was incubated with anti-Flag M2-agarose affinity gel (Sigma Chemical Co., St Louis, MO) for 12 h at 4°C with rotation in TNE buffer. The beads were washed four times with TNE buffer. After centrifugation, total RNA was extracted from the precipitate using a RNeasy Miniprep Kit (Qiagen). Total mRNAs were reverse transcribed and amplified by PCR using the ThermoScript RT–PCR System (Invitrogen). The following YB-1- and β-actin-specific primer pairs were used: the forward primers were 5'-ACCACAGTATTCCAACCC-TCCTG-3' (YB-1) and 5'-CTGGCACCACACCTTCTA-CAATG-3' (β-actin) and the reverse primers were 5'-ATC-TTCTTCATTGCCGTCCTCTC-3' (YB-1) and 5'-ATAGC-AACGTACATGGCTGGGG-3' (β-actin). The RT–PCR

amplification products were analyzed by 2% agarose gel electrophoresis.

RNA band shift assays

The RNA electrophoretic mobility shift assay (REMSA) was carried out according to established techniques (29). Briefly, ^{32}P-labeled YB-1 5'-UTR probe was transcribed *in vitro* from the plasmid pT7blue3, which contains a sequence corresponding to the 5'-UTR of YB-1. An aliquot of 2 µg of the linearized plasmid was transcribed *in vitro* by T7 RNA polymerase in the presence of [α-^{32}P]UTP. The DNA template was removed by digestion with DNase I and the YB-1 5'-UTR probe was then extracted by column chromatography. To form RNA–protein complexes, 1–10 µg of cytoplasmic protein or the indicated amount of purified GST–YB-1 was incubated with ^{32}P-labeled YB-1 5'-UTR probe at 25°C for 15 min. Next, the samples underwent electrophoresis through a 4% non-denaturing polyacrylamide gel (polyacrylamide:bisacrylamide, 80:1) in Tris–borate buffer. For the supershift experiments, 2 µg of the YB-1 antibody was incubated with cytoplasmic protein or purified GST–YB-1 at 25°C for 5 min before adding the ^{32}P-labeled YB-1 5'-UTR probe. The gels were dried, visualized and then quantified as described above for the primer extension analysis.

In vitro transcription and translation

An aliquot of 2 µg of plasmid pT7Blue3, which encodes both luciferase cDNA ligated to the YB-1 mRNA 5'-UTR region (YB-1 5'-UTR–luciferase) as well as luciferase cDNA not ligated to the YB-1 mRNA 5'-UTR region (luciferase) (see Fig. 2A), was transcribed *in vitro* using an *In vitro* Transcription System (Promega). The DNA template was removed by digestion with DNase I and the RNA was purified by phenol/chloroform extraction. The integrity of the RNA was then examined using an Agilent 2000 Bioanalizer (Yokogawa, Osaka, Japan). Then, 50 ng of each RNA was translated using a rabbit reticulocyte lysate system (Promega). The luciferase assay was performed after incubation for 1.5 h at 30°C as described previously (18). To characterize the translation inhibition of each of the reporter constructs, the indicated amounts of GST fusion proteins were added to the rabbit reticulocyte lysate system either initially or after 10 min of incubation using 50 ng of the RNA constructs. The *in vitro* translation was performed for 30 min at 30°C and the translation activity of each experiment was measured. Data are shown as the means ± SD from three independent experiments.

Northern blot analysis

To detect *in vitro* transcribed RNA, a luciferase cDNA was used as the probe. The luciferase cDNA was obtained by digesting the pGL3-Basic vector (Promega) with NarI and XbaI. Reaction mixtures (20 µl, as described above) containing 50 ng of *in vitro* transcribed RNA were preincubated or treated after 10 min incubation with 5 pmol of GST–YB-1. After incubation for 30 min in a rabbit reticulocyte lysate system, total RNA was extracted using an RNeasy Miniprep Kit (Qiagen). RNA samples (0.5 µg/lane) were separated on a 1% formaldehyde–agarose gel and transferred onto Biodyne B membranes (Pall, Port Washington, NY). The membrane was

prehybridized and hybridized with [α-^{32}P]dCTP-labeled probe. Radioactivity was analyzed by autoradiograpy.

Transfections and luciferase assays

Cells underwent transient transfection using the LipofectAMINE method. Human lung cancer H1299 cells were plated at a density of 1 × 10^5 cells/35 mm well the day before transfection. At ~80–90% confluence, the cells were transfected with 1 μg of reporter plasmid DNA or control vector using LipofectAMINE 2000 reagent (Invitrogen). Three hours later, the cells were washed twice with PBS and placed in fresh medium. Twenty-four hours post-transfection, the luciferase activity was measured as described below. The luciferase activity of the transfected cells was measured using the Dual Luciferase Assay System (Promega). Briefly, cells were lysed with 250 μl of 1× Passive Lysis Buffer (Promega). After a brief centrifugation, 10 μl of each supernatant was assayed for luciferase activity. Light emission was measured for 15 s with a luminometer. To standardize translation efficiency, the relative luciferase activity was expressed as the ratio of downstream cistron expression to upstream cistron expression (luciferase/EYFP). The fluorescence of enhanced yellow fluorescent protein (EYFP) was excited at 488 nm and measured for 1 s using a fluorometer.

RESULTS

Analysis of the multiple transcription initiation sites of the YB-1 gene

To investigate the mechanisms of translational control regulating YB-1 protein levels, the possible involvement of YB-1 mRNA was investigated. The 5′-UTR has a high G+C content (61%), suggesting that it could assume a high level of secondary structure *in vivo*. Computer modeling of potential secondary structures suggested that structures with free energies (ΔG values) lower than −190 kcal/mol could be formed (data not shown). We previously identified several transcription initiation sites for the YB-1 gene in KB3-1 and T24 cells (28). To compare the major transcription initiation sites in other cell lines, primer extension analysis was performed (Fig. 1A). The lung cancer cell line H1299 and endothelial cells (HUVECs), were compared with KB3-1 cells. The transcription initiation sites (*) were identical in all of the cells and eight transcripts (*1–*8) were observed in the region mapped to +1 to +197 (Fig. 1B). The ratio of the transcripts differed in each cell line. The proportion of transcripts starting at initiation site *1 was significantly higher in HUVECs, compared to the other cancer cells. These results suggest that the multiple transcription start sites of YB-1 mRNA observed in cell lines may be involved in the regulation of YB-1 protein expression.

The 5′-UTR of YB-1 mRNA increases the expression of a luciferase reporter *in vitro* and *in vivo*

To study the possible involvement of the 5′-UTR of YB-1 mRNA in translation control, we generated two types of reporter constructs. (i) Those containing luciferase cDNA ligated to the full-length 5′-UTR of YB-1 gene (pT7-YB5′-1) and (ii) the non-ligated luciferase control construct (pT7) (Fig. 2A). The predominant 5′ transcription initiation site is
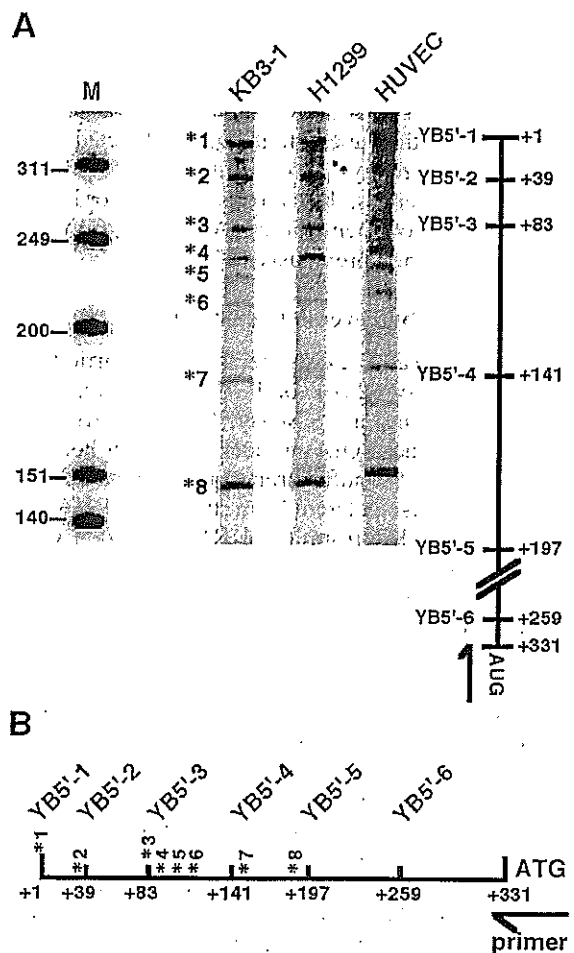

Figure 1. Analysis of the transcription start site of the YB-1 gene by primer extension assay. (A) Primer extension assay. Hybridization of the primer extension was performed with 5′-labeled oligonucleotide and 1 μg poly(A)$^+$ RNA of each cell line. The markers shown on the left are end-labeled HinfI fragments of φ-X174 DNA. Black asterisks (*) indicate transcription initiation start sites. The 5′ furthest transcription initiation start site is indicated as +1 and the first AUG codon is indicated at +331. The arrow indicates the position of the primer that was used for extension. The primer hybridizes the sense strand between nucleotides +317 and +337 of the YB-1 gene. (B) Schematic distribution of the transcription initiation site in the YB-1 5′-UTR.

designated +1 in the text and figures. Additional experiments were performed to investigate the regulatory region of the 5′-UTR of YB-1 mRNA. A total of five serial deletion mutants (pT7-YB-5′-2–pT7-YB5′-6) were constructed, each corresponding to a particular transcription initiation site. We first cloned transcripts *1, *2, *4, *7 and *8, which were each expressed at up to 10% of the total. These transcripts corresponded to YB5′-1–YB5′-5. We made an additional probe, YB5′-6, for detection of the shortest 5′-UTR fragment of YB-1. *In vitro* transcription reactions were performed on each of these constructs using T7 RNA polymerase and the integrity of the transcripts was confirmed by gel electrophoresis (data not shown). The differences in the transcript sizes were consistent with the length of the 5′-UTR (Fig. 2A). Equal amounts of the transcripts were translated in rabbit reticulocyte lysate and the luciferase activity was measured. The presence of the full-length 5′-UTR of YB-1 mRNA by itself
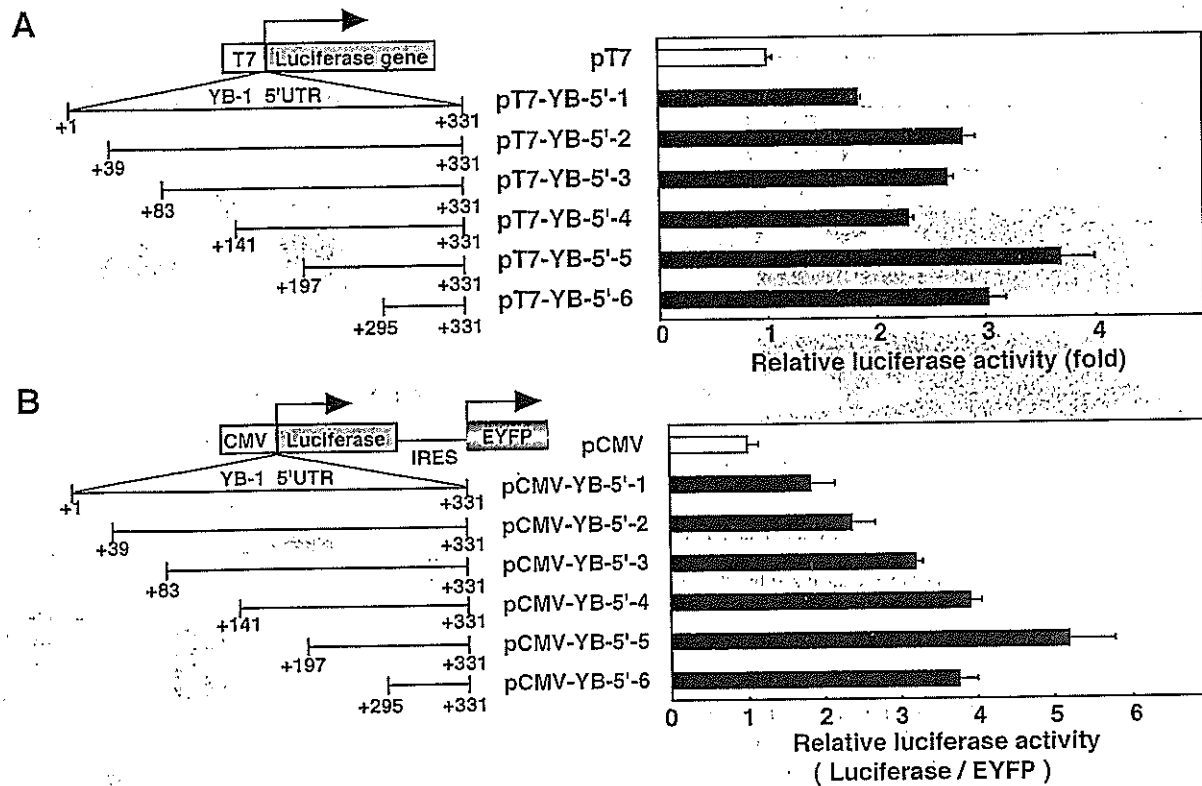
A

T7	Luciferase gene		pT7
YB-1 5'UTR			pT7-YB-5'-1
+1		+331	pT7-YB-5'-2
+39		+331	pT7-YB-5'-3
+83		+331	pT7-YB-5'-4
+141		+331	pT7-YB-5'-5
+197		+331	pT7-YB-5'-6
+295		+331	

Relative luciferase activity (fold)

B

CMV	Luciferase	IRES	EYFP	pCMV
YB-1 5'UTR				pCMV-YB-5'-1
+1			+331	pCMV-YB-5'-2
+39			+331	pCMV-YB-5'-3
+83			+331	pCMV-YB-5'-4
+141			+331	pCMV-YB-5'-5
+197			+331	pCMV-YB-5'-6
+295			+331	

Relative luciferase activity
(Luciferase / EYFP)

Figure 2. Effect of deletions of the 5'-UTR of YB-1 on the expression of a luciferase reporter *in vitro* and *in vivo*. (A) Schematic representation of the reporter constructs containing luciferase cDNA ligated to the YB-1 5'-UTR region (pT7-YB5'-1–pT7-YB5'-6) fragments of various sizes, as well as the non-ligated control construct (pT7). The YB-1 5'-UTR region (pT7-YB5'-1–pT7-YB5'-6) fragments were enlarged to show the limits of regions of the 5'-UTR of YB-1. The right-angled arrow denotes the start site and direction of transcription. Each of the reporter plasmids was transcribed *in vitro* in a reaction driven by T7 RNA polymerase and then 50 ng of the RNA constructs were translated using the rabbit reticulocyte lysate system. The luciferase assay was performed after incubation for 90 min at 30°C. (B) Schematic representation of the bicistronic reporter constructs containing luciferase cDNA ligated to the YB-1 5'-UTR region (pCMV-YB5'-1–pCMV-YB5'-6) fragments of various sizes, as well as the non-ligated control construct (pCMV). Each of the reporter plasmids was transcribed under the control of the human cytomegalovirus early promoter (CMV). The right-angled arrow indicates the start site and direction of translation. H1299 cells were transfected with these constructs and their luciferase activities were measured. To standardize the translation efficiency, relative luciferase activity was expressed as the ratio of downstream cistron expression to upstream cistron expression (luciferase/EYFP). Data are shown as the means ± SD (error bars) of three independent experiments.

increased the level of luciferase activity ~2-fold relative to that of the control mRNA (pT7). Furthermore, each of the YB-1 5'-UTR deletion constructs showed higher translational activity than did the pT7 control. Of all the constructs, pT7-YB5'-5 showed the highest activity, which was ~4-fold greater than that of the control construct (Fig. 2A).

We next determined whether a similar increase in activity could also be induced in cultured cells by the 5'-UTR. We constructed eukaryotic expression vectors containing luciferase cDNA ligated to various regions of the YB-1 5'-UTR (pCMV-YB5'-1–pCMV-YB5'-6), as described for the *in vitro* experiment (Fig. 2A). The reporter constructs were transcribed under control of the human cytomegalovirus early promoter. After transfection into a H1299 lung carcinoma cell line, the levels of luciferase activity were compared (Fig. 2B). As observed with the *in vitro* translation assays (Fig. 2A), the YB-1 5'-UTR increased the level of luciferase expression. The full-length YB-1 5'-UTR by itself increased luciferase activity ~2-fold compared to the control construct (pCMV). Furthermore, pCMV-YB5'-5 also showed the highest activity, which was 4–5-fold that of the control. The mRNAs encoded

by the reporter constructs were equally expressed, as determined by northern blot analysis (data not shown). These results suggest that the YB-1 5'-UTR enables more efficient translation of mRNA *in vitro* and *in vivo* and the short-length construct (YB5'-5) facilitates the most efficient translational activity.

YB-1 binds its own mRNA in the cytoplasm

YB-1, which posseses RNA binding activity (26), has been reported to be involved in translational regulation and in the regulation of mRNA stability (16). In order to identify whether or not YB-1 protein interacts with its own mRNA in the cytoplasm, we performed RT–PCR using mRNA isolated by co-immunoprecipitation with YB-1 immunoprecipitant (Fig. 3). Flag–YB-1 or an empty Flag expression vector were transfected into an H1299 lung carcinoma cell line. After 48 h, cells were lysed and YB-1 proteins were immunoprecipitated with Flag antibody. The mRNA was purified after immunoprecipitation and amplified by RT–PCR using YB-1- and β-actin-specific primers. YB-1 mRNA and β-actin mRNA were both expressed in the H1299 cell lines (Fig. 3, lane 4).
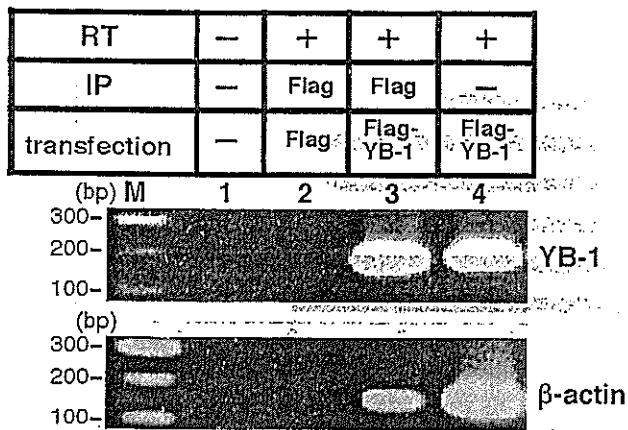
Figure 3. YB-1 interacts with its own mRNA in the cytoplasm. Flag–YB-1 or Flag expression vector was transfected into H1299 cells. After 48 h, cells were lysed and the YB-1 proteins were immunoprecipitated (IP) with Flag antibody. The mRNA were purified after immunoprecipitation and amplified by RT–PCR with YB-1- (upper panel) and β-actin-specific (lower panel) primers. The RT–PCR amplification products were analyzed by 2% agarose gel electrophoresis.

Flag–YB-1 immunoprecipitates contained YB-1 mRNA (Fig. 3, lane 3), while the control Flag immunoprecipitate did not (Fig. 3, lane 2). Furthermore, the YB-1 immunoprecipitate also contained β-actin mRNA (Fig. 3, lane 3). These data provide evidence that YB-1 interacts with its own mRNA and β-actin mRNA in the cytoplasm of cultured cells.

YB-1 binds to the 5'-UTR of its cognate mRNA through a C-terminal domain

We next investigated the interaction of YB-1 protein with the 5'-UTR of its own mRNA *in vitro*. An RNA gel shift assay (REMSA) was performed by using KB3-1 and H1299 cell lysates and an *in vitro* synthesized mRNA probe corresponding to the full-length 5'-UTR of YB-1 mRNA (Fig. 4A). YB-1 5'-UTR formed an RNA–protein complex with lysates made using KB3-1 and H1299 cell lysates (Fig. 4A, lanes 2–4 and 8–10). The presence of endogenous YB-1 protein in the major complex was confirmed by the ability of a YB-1-specific antibody to supershift most of the complex (Fig. 4A, lanes 6 and 12). To determine whether YB-1 protein is able to directly interact with the 5'-UTR of YB-1 mRNA, we performed a REMSA using purified recombinant YB-1 (Fig. 4B). Recombinant YB-1 also clearly bound to the 5'-UTR of YB-1 mRNA (Fig. 4B, lanes 5–7), while control GST protein did not (Fig. 4B, lanes 2–4). The interaction with YB-1 protein was also confirmed by the use of YB-1-specific antibody (Fig. 4B, lanes 10 and 11).

We previously observed that rabbit p50, a homolog of human YB-1 protein, was present in rabbit reticulocyte lysate. To assess the nature of the rabbit p50 interaction with the 5'-UTR of YB-1 mRNA, we performed a REMSA using rabbit reticulocyte lysate (Fig. 4C). When added to rabbit reticulocyte lysate, the YB-1 5'-UTR formed an RNA–protein complex (Fig. 4C, lanes 2 and 3). The presence of rabbit p50 in the major complex was confirmed by the ability of a YB-1-specific antibody to supershift most of the complex (Fig. 4C, lane 5).
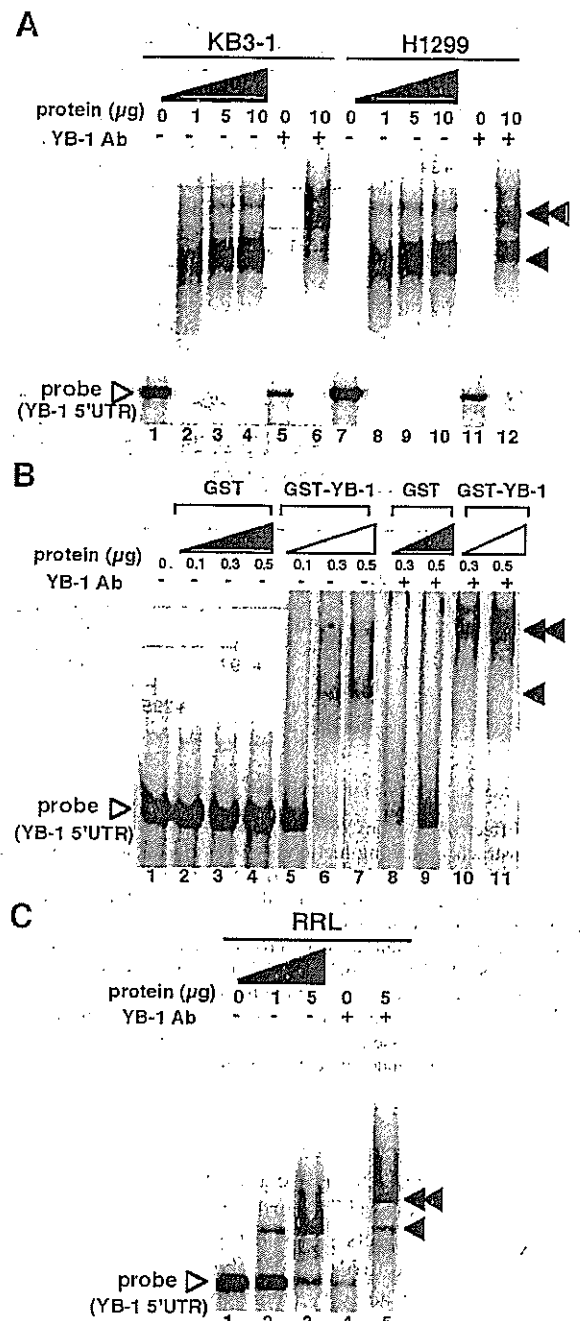

Figure 4. YB-1 binds to the 5'-UTR region of YB-1mRNA. (A) Endogenous YB-1 protein binds to the 5'-UTR region of YB-1 mRNA in the cytoplasm. The indicated amounts of KB3-1 and H1299 cell lysate were incubated with 32P-labeled YB-1 5'-UTR RNA at 25°C for 15 min. An aliquot of 2 µg YB-1-specific antibody (Ab) was added to lanes 5, 6, 11 and 12. An arrow indicates the YB-1/YB-1 5'-UTR RNA complex and a double-headed arrow indicates supershifted complexes. (B) Purified YB-1 binds to YB-1 5'-UTR RNA. The indicated amount of GST or GST–YB-1 fusion was incubated with 32P-labeled YB-1 5'-UTR RNA at 25°C for 15 min. An aliquot of 2 µg YB-1-specific antibody (Ab) was added to lanes 8–11. An arrow indicates the YB-1/YB-1 5'-UTR RNA complex and a double-headed arrow indicates supershifted complexes. (C) Rabbit YB-1 (p50) binds to the 5'-UTR region of YB-1 mRNA in the *in vitro* translation system. The indicated amounts of rabbit reticulocyte lysate (RRL) were incubated with 32P-labeled YB-1 5'-UTR RNA at 25°C for 15 min. An aliquot of 2 µg YB-1-specific antibody (Ab) was added to lanes 4 and 5. An arrow indicates the rabbit YB-1/YB-1 5'-UTR RNA complex and a double-headed arrow indicates supershifted complexes. YB-1/YB-1 5'-UTR RNA probe complexes were separated as described above.
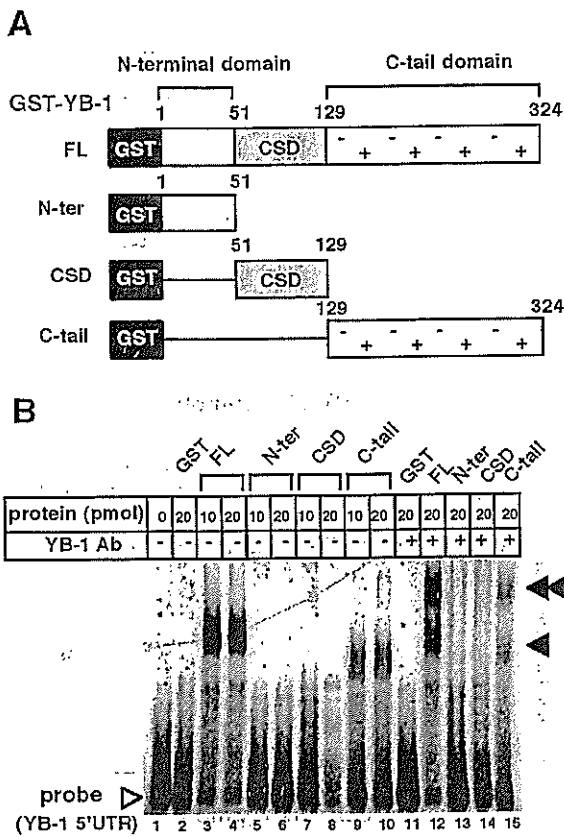
Figure 5. Identification of the YB-1 5'-UTR binding domain in YB-1. (A) Schematic illustration of the GST-YB-1 deletion mutants used in this study. CSD indicates the cold·shock domain. (B) REMSA assay. The indicated amount of each GST-YB-1 deletion mutant and GST was incubated with [32P]-labeled YB-1 5'-UTR RNA at 25°C for 15 min. An aliquot of 2 µg YB-1-specific antibody (Ab) was added to lanes 11-15. An arrow indicates the YB-1/YB-1 5'-UTR RNA complex and a double-headed arrow indicates supershifted complexes. YB-1 mutant/YB-1 5'-UTR RNA probe complexes were separated using 4% native polyacrylamide gels.
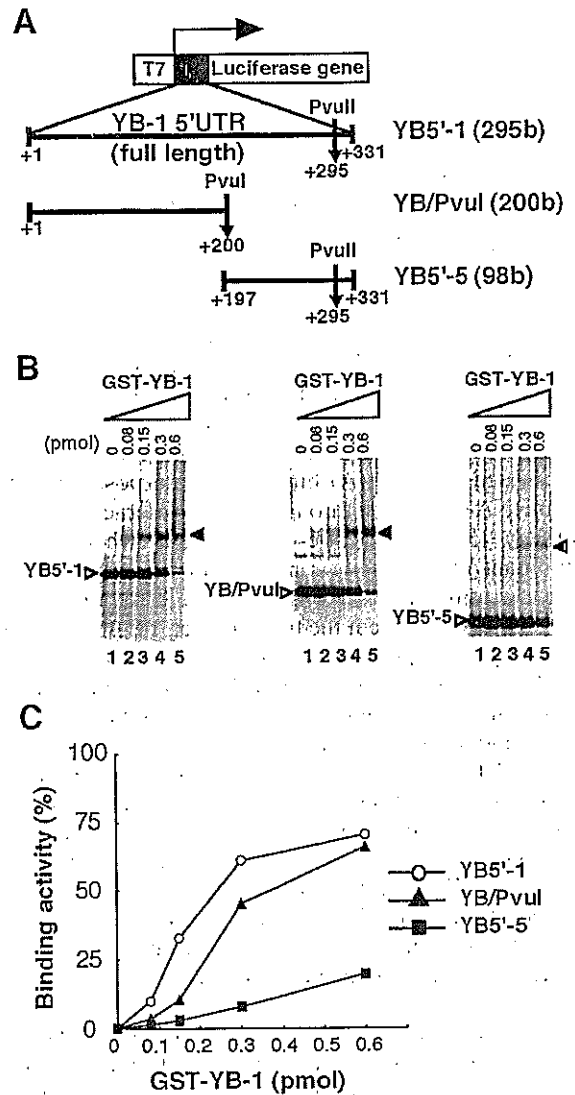

Figure 6. Identification of the YB-1 binding region in YB-1 5'-UTR mRNA. (A) Schematic illustration of YB-1 5'-UTR deletion constructs used as the probe in a REMSA. To produce RNA of defined length, restriction enzyme (PvuI or PvuII) was used to linearize the DNA templates. (B) REMSA. The indicated amount of GST-YB-1 fusion was incubated with each [32P]-labeled YB-1 5'-UTR deletion mRNA at 25°C for 15 min. An arrow indicates the YB-1/YB-1 5'-UTR RNA complex. YB-1/YB-1 5'-UTR RNA probe complexes were separated using 4% native polyacrylamide gels. (C) Kinetic analysis of GST-YB-1 binding to YB5'-1, YB/PvuI and YB5'-5 probes. The GST-YB-1 binding activity to YB-1 5'-UTR fragments identical to that shown in (B) were quantified by monitoring amounts of the binding complexes.

YB-1 protein consists of three major domains, each of which has the potential to bind nucleic acids (30): the alanine/proline-rich N-terminal domain, the highly conserved nucleic acid binding domain and the C-tail domain. To identify which domains of YB-1 protein are responsible for this interaction, we performed a REMSA using a series of GST fusion proteins containing either full-length YB-1 (FL) or the mutant derivatives GST-YB-1 N-ter, CSD and C-tail (Fig. 5A). A strong interaction was observed using the full-length YB-1 (Fig. 5B, lanes 3 and 4) and the C-tail of YB-1 (Fig. 5B, lanes 9 and 10). The central CSD, containing the RNP1 motif, was not able to bind to the 5'-UTR of YB-1 mRNA (Fig. 5B, lanes 7 and 8). The binding activity of both constructs resulted in the appearance of a supershifted band in the presence of a YB-1-specific antibody that recognizes the C-terminal tail of YB-1 (Fig. 5B, lanes 12 and 15). These results suggest that the C-tail domain of YB-1 protein interacts with the 5'-UTR of its own mRNA, independent of other domains.

Identification of the YB-1 binding region in the 5'-UTR of YB-1 mRNA

To identify which region of the YB-1 mRNA 5'-UTR interacts with YB-1 protein, we performed a REMSA using probes corresponding to YB5'-1 (295 bases), YB/PvuI (200 bases) and YB5'-5 (98 bases), as defined in Figure 1B (Fig. 6A). All three constructs formed RNA–protein complexes in a dose-dependent manner. However, the affinity of YB-1 for the three probes differed. When GST-YB-1 was added to the full-length YB-1 5'-UTR (YB5'-1) and YB/PvuI, a retarded complex was clearly visible (Fig. 6B). When 0.6 pmol of GST-YB-1 was added, up to 60% of the YB5'-1 and YB/PvuI probes were bound to GST-YB-1 (Fig. 6C). However, little retarded band was observed using the deleted YB5'-5 probe (Fig. 6B) when the same amount of YB-1 was used. When 0.6 pmol of YB-1 was added to YB5'-5, <20% YB5'-5 was

bound. These results indicate that YB-1 protein binds to the full-length and the first half of the YB-1 5'-UTR more efficiently than a construct containing the second half of the YB-1 5'-UTR, YB5'-5.

Recombinant YB-1 protein decreases translation through its own 5'-UTR mRNA element *in vitro*

As shown in the REMSA, we observed higher affinity binding to the full-length 5'-UTR of YB-1 mRNA with recombinant YB-1. It has been shown that inhibitory concentrations of YB-1 suppressed the interaction with initiation complexes such as eIF4E, eIF4A and eIF4B, and these concentrations inhibited translation at the stage of initiation (17,31). YB-1 has also been reported to affect the translation of both cap^+ $poly(A)^+$ and cap^- $poly(A)^-$ mRNAs (1,2). Skabkina *et al.* also demonstrated that poly(A) binding protein positively affects YB-1 mRNA translation through specific interaction with YB-1 mRNA (32). Thus, we investigated whether or not recombinant YB-1 inhibits translation in a cell-free translation system by using the full-length and deleted YB-1 5'-UTR constructs. In this system the transcript of YB-1 5'-UTR were neither capped nor polyadenylated. We therefore excluded the possibility that binding between YB-1 protein and translation initiation and elongation factors such as cap binding protein or poly(A) binding protein was involved. To test the functional activity of YB-1 protein, recombinant YB-1 protein was added to the *in vitro* translation system. *In vitro* transcripts from pT7, pT7-YB5'-1 and pT7-YB5'-5 were translated in a rabbit reticulocyte lysate system in the absence or presence of recombinant YB-1 protein and the luciferase activity was measured. As shown in Figure 7A, the addition of YB-1 at the start of translation inhibited the translation of all three constructs in a dose-dependent manner to a maximum of ~60%.

Next, we measured the kinetics of luciferase translation in a rabbit reticulocyte lysate. After the first 10 min, almost 10% of translation had occurred and translation efficiency was saturated after 60 min (data not shown). To investigate the inhibitory effects of YB-1 protein on translation, we added various amounts of YB-1 protein 10 min after translation was started (Fig. 7B). The addition of YB-1 10 min after translation inhibited the luciferase activity in a dose-dependent manner when the construct containing the full-length 5'-UTR (pT7-YB5'-1) was used; this reduction amounted to ~50% with 5 pmol of YB-1, implying that only 10% of the inhibition results from translation that could have occurred in the first 10 min of the 30 min reaction. On the other hand, little YB-1-dependent repression of translation was seen using pT7-YB5'-5 and pT7-luciferase. These results suggested that pretreated YB-1 inhibited translation of all constructs and the addition of YB-1 after translation also specifically inhibited translation of the construct with the full-length 5'-UTR.

YB-1 protein was also involved in stabilization of mRNA in rabbit reticulocyte system. We confirmed the integrity of the *in vitro* transcribed mRNA by northern blot analysis before and after treatment with YB-1 (Fig. 7C). No change in transcribed RNA stability was observed with up to 30 min incubation in rabbit reticulocyte system (Fig. 7C, lanes 1–3, 4–6 and 7–9).
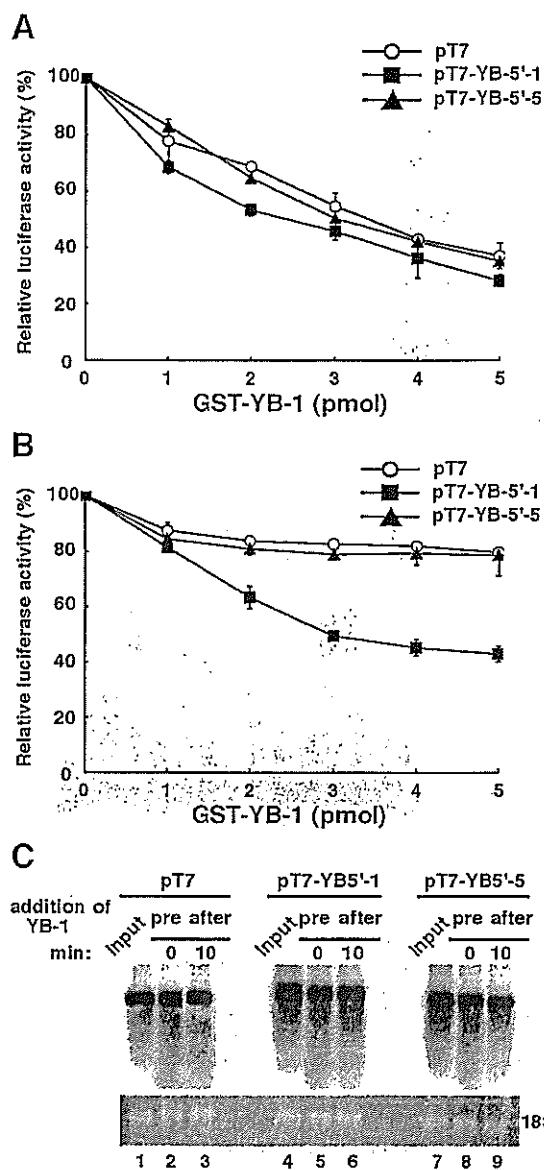
Figure 7. Characterization of translation inhibition by YB-1 protein through its own 5'-UTR mRNA element *in vitro*. The indicated amounts of GST–YB-1 fusion were added to a rabbit reticulocyte lysate system either initially (A) or after 10 min incubation (B) using 50 ng of the RNA constructs described in Figure 2A. The *in vitro* translation was performed for 30 min at 30°C and the translational products were directly used for the luciferase assay. Data are shown as means ± SD (error bars) of three independent experiments. (C) The effect of YB-1 on mRNA stability was examined. An aliquot of 50 ng of *in vitro* transcribed RNA constructs was preincubated (pre) or treated after 10 min incubation (after) with 5 pmol of GST–YB-1. After incubation for 30 min in a rabbit reticulocyte lysate system, the RNA was detected by northern blot hybridization. Lanes 1, 4 and 7 (Input) show the *in vitro* transcribed RNA constructs isolated from the exact at 0 min incubation.

Recombinant YB-1 protein did not decrease translation through a mutant 5'-UTR element

Next, we focused on the possible secondary structure of the full-length 5'-UTR of YB-1 mRNA. In general, the stem–loop structure found in 5'-UTRs of mRNAs affect their translational efficiency as, for example, in the ferritin 5'-UTR (18). In

the YB-1 5′-UTR, we observed three regions which are predicted to form possible stem–loop structures. To investigate the translational regulatory region of the 5′-UTR of YB-1 mRNA, we constructed three mutants which disrupt the stem–loop structure. These contained internal mutations (mut1–mut3) which we have designated using the nucleotide at the 5′ end corresponding to the transcription initiation site (*) (Fig. 8A). To characterize the translational inhibition observed with the addition of YB-1, we performed cell-free translational assays using mutants of the above constructs of the YB-1 5′-UTR fused to luciferase mRNA (Fig. 8B and C). The addition of YB-1 at the start of translation resulted in up to a 60% inhibition of luciferase activity in a dose-dependent manner, in the case of both the mutant and full-length 5′-UTR (Fig. 8B). However, no repression of the luciferase activity of these mutant constructs (pT7-YBM1–pT7-YBM3) was observed when YB-1 was added 10 min after translation was initiated (Fig. 8C). These results suggest that YB-1 protein regulates the translation of all constructs and also that it specifically inhibits translation when the 5′-UTR of YB-1 mRNA is present. Our findings are consistent with a model in which YB-1 protein specifically binds to the full-length 5′-UTR of YB-1 mRNA and inhibits its own translation *in vitro*.

DISCUSSION

In this report, our data demonstrate that YB-1 protein not only binds to an RNA containing the human YB-1 5′-UTR *in vitro* and *in vivo*, but that it also exhibits functional activity by specifically inhibiting the translation of a YB-1 5′-UTR–luciferase reporter mRNA in rabbit reticulocyte lysate assays. The multifunctional protein YB-1 was first identified as a transcription factor which binds to the Y-box of the MHC class II promoter sequence (33). Recent studies have demonstrated that YB-1 regulates gene expression not only at the level of transcription, but also at the level of mRNA translation. Considering these characteristics, it is possible that YB-1 might regulate its own expression at the translational level.

The present paper provides evidence that the translational control of YB-1 expression is mediated via the 5′-UTR of YB-1 mRNA. Global control of translational efficiency can be achieved by regulating the phosphorylation state of an array of initiation factors (such as eIF2 and eIF4E). However, control of translational initiation on an individual mRNA is determined primarily by its nucleotide sequence and the secondary structure of the regulatory protein. The 5′-UTR plays a particularly important role in the regulation of translation initiation via an interaction with RNA binding proteins or by secondary structure formation, which hinders the activity of the translational machinery. Analysis of the 5′-UTR sequence of YB-1 (331 bases in length) revealed several important features that are known to influence translation.

Many *in vitro* and *in vivo* studies have shown that mRNAs with a high likelihood of forming a stable secondary structure in the 5′-UTR tend to be inefficiently translated (34). However, in both *in vitro* and *in vivo* experiments we found that the 5′-UTR sequence of YB-1 mRNA, with its high G+C content, acts as a potent translational enhancer. The full-length 5′-UTR of YB-1 mRNA increased the level of translation activity and cloning a 134 base region of the YB-1 5′-UTR
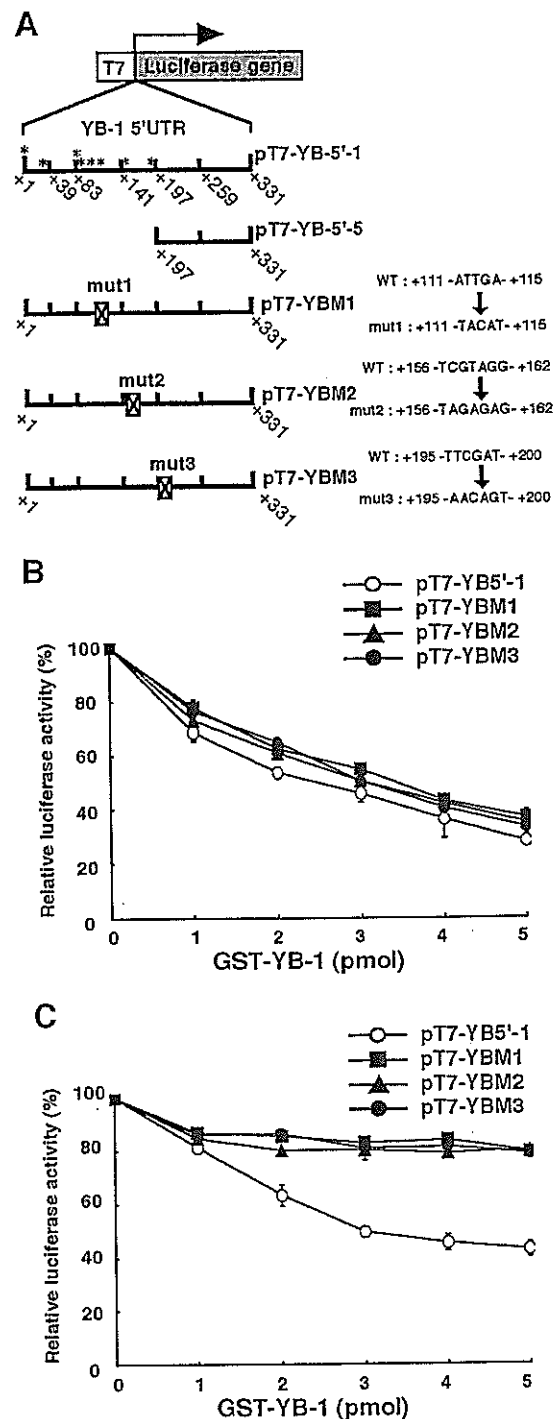

Figure 8. Effect of mutation of the YB-1 5′-UTR mRNA element on translation. (A) Schematic representation of the YB-1 5′-UTR–luciferase fusion reporter constructs used in this study. pT7-YBM1–3 containing an internal mutation (mut1–3), designated by the nucleotide at the 5′ end that corresponded to the transcription initiation sites (*) (see Fig. 1A). (B and C) The characterization of translation inhibition by each reporter construct was compared to that of the other constructs. The indicated amounts of GST–YB-1 fusion were added to a rabbit reticulocyte lysate system either initially (B) or after 10 min incubation (C) using 50 ng of the RNA constructs described in (A). The *in vitro* translation was performed for 30 min at 30°C and the translation activity of each experiment was measured. Data are shown as the means ± SD (error bars) of three independent experiments.

upstream of the coding initiation sequence was also associated with a significant increase in translation activity (Fig. 2A and B). This region might therefore be crucial for the translation activity of the 5′-UTR of YB-1 mRNA. These data suggest that elements within the 5′-UTR of YB-1 mRNA can act as enhancers of mRNA translation.

According to the scanning mechanism postulated for translation initiation, the small (40S) ribosomal subunit enters at the 5′ end of the mRNA and migrates linearly, stopping when the first AUG codon is reached (35). Nekrasov *et al.* (31) demonstrated that YB-1 protein inhibited the initiation step of translation, but did not inhibit the elongation step. Rabbit YB-1 displays both RNA melting and annealing activities in a dose-dependent manner; a relatively low amount of YB-1 promotes the formation of RNA duplexes, whereas an excess of YB-1 causes unwinding of double-stranded forms (36). It is also possible that YB-1 additionally facilitates movement of the small ribosomal subunit to the initiation codon complexed with initiator tRNA^{Met}. This may occur up to the initiation codon by YB-1 melting the secondary structure in the mRNA 5′-UTR (31).

In this study, we observed that YB-1 inhibited the translation of all of the transcripts of YB-1 5′-UTR that lacked sequence specificity, suggesting that YB-1 reduced the translation efficiency of 5′-UTR elements, either at the initiation step or by binding directly to the whole RNA. These results provided evidence that YB-1 might block the first step in mRNA recruitment into translation initiation.

The addition of YB-1 10 min after initiation inhibited translational activity only when the full-length 5′-UTR of YB-1 mRNA was present (Figs 7B and 8C). No repression of the luciferase activity of the deleted or mutant constructs (pT7-YBM1–pT7-YBM3) (with disrupted stem–loop structures) was observed. We also observed that YB-1 binds to the first half-region of YB-1 5′ UTR RNA more efficiently than does the latter half construct comprising the second half (Fig. 7). The affinity of YB-1 for the mutated probes was the same as for the wild-type YB5′-1 probes (data not shown). These results suggest that ribosome scanning or ongoing translation might be required for repression by YB-1 when the full-length YB-1 5′-UTR was used. And when mutated and deleted probe were used, YB-1 did not affect ribosomal scanning due to alterations in the secondary structure of the YB-1 5′ UTR. The precise secondary structures of the YB-1 5′-UTR remains to be determined.

By combining the results presented here with previous data, we are able to describe the effect of YB-1 on translation. First, YB-1 blocks the initial step in mRNA recruitment to translation initiation, YB-1 then turns off the interaction of the mRNA with eIF4F (31). Second, YB-1 promotes movement of the small ribosomal subunit within the complex by melting the mRNA 5′-UTR secondary structure, suggesting that YB-1 affects ribosome scanning through the 5′-UTR.

The 5′-UTR plays an important role in regulation of the expression of a number of genes. For instance, the UTRs of mRNAs encoding heat shock proteins (HSPs), known to protect cells against a wide variety of stresses, including heat shock, viral infection and exposure to oxidative free radicals and toxic metal ions (37), have been reported to contain elements important for the post-transcriptional regulation of these key components of the stress response (38,39).

YB-1 has been reported to be involved in the regulation of stress-inducible target genes (40,41), suggesting that YB-1 itself is a stress-activated protein. YB-1 mRNA accumulates in cells when they are treated with UV irradiation or anticancer agents. We previously reported that c-myc and p73 activate YB-1 transcription and may regulate important biological processes via their effects on YB-1 gene expression (42). Additional experiments are needed to confirm the role of YB-1 in regulating translation *in vivo* under various stress conditions. It would be of interest to determine how the 5′-UTR of YB-1 mRNA affects the translation of YB-1 in response to extracellular stimuli or environmental stress.

The translational control of gene expression has been identified as an important regulatory mechanism for many gene products involved in the regulation of proliferation [e.g. c-mos (43), FGF-2 (44,45), PDGF-B (46), p27 (47) and cdk4 (48)]. The tumor suppressor p53 has been implicated in the translational regulation of both p53 and cdk4 mRNAs (49). Similarly, several distinct components of the translation apparatus have been shown to be deregulated or overexpressed in human tumors. So, it is of note that the level of the YB-1 mRNA from the 5′ furthest transcription initiation start site was significantly higher in HUVEC cells, compared to the other cancer cell lines examined (Fig. 1). The deregulation of the translational control of YB-1 might therefore also play a role in tumor progression.

In conclusion, YB-1 protein binds to an RNA containing the 5′-UTR of human YB-1 mRNA *in vitro* and *in vivo* and exhibits functional activity by inhibiting the translation of a YB-1 5′-UTR–luciferase reporter mRNA through the aid of its own 5′-UTR mRNA. We propose a model in which YB-1 protein autoregulates its own translation by binding to the 5′-UTR of its own mRNA. Our results support a *trans*-acting repressor hypothesis, in which a repressor protein specifically binds to its own 5′-UTR element, resulting in translational repression and the maintenance of constant protein levels.

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