3．研究成果の刊行物・別刷

Special Review

テロメアを維持する仕組み
― ATMファミリータンパク質によるゲノム統合性維持機構

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別刷

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テロメアは染色体の必須エレメントとしてゲノム情報の維持に重要な役割を果たしている．テロメアの機能は構成要素であるテロメア反復配列と，そこに局在する複数のタンパク質との複合体構造により担われている．真核生物でよく保存されたATMファミリータンパク質は，テロメア機能の制御に関与し，その欠損は種々の染色体異常を引き起こす．ATM関連因子が，テロメア配列自体を制御しているのみならず，テロメア高次構造をも制御している可能性が示唆されてきている．

◆key words
テロメア，テロメラーゼ，ATMファミリータンパク質，非相同組換え，サイレンシング，ヘテロクロマチン

はじめに：テロメアDNAはなぜ不安定なのか？

遺伝情報の子孫への正確な伝達は，ゲノム情報の正確な複製と，その情報の娘細胞への正確な分配に依存する．真核生物の染色体DNAは線状であり，それゆえに末端での複製において潜在的問題を抱えている．その第一点はDNAポリメラーゼがRNAをプライマーとして要求することに起因する，いわゆる末端複製問題である．また，真の染色体末端は，特殊な構造を形成することで損傷により生ずる二重鎖切断末端となんらかの方法で区別されているはずであるが，複製過程でその構造が一時的にしろ失われる結果，核内のDNA消化酵素による攻撃を受け末端が分解されてしまう可能性がある．さらに，末端を構成するDNAは多くの生物種において反復配列に富んでおり，組換えにより末端近傍の配列を失う危険性を秘めている．

真核生物は染色体末端にテロメアと呼ばれる特殊化したDNA-タンパク質複合体構造を形成することで，それらの問題を少なくとも部分的には解決し，染色体が持つゲノム情報を維持している．本稿では，テロメアの維持機構をDNA一次配列の維持，DNA-タンパク質複合体として形成される高次構造の維持という側面から解説するとともに，ヒト染色体不安定性症候群の原因遺伝子*ATM*とそのファミリー遺伝子産物のテロメアの構造維持への関与，その欠損がもたらすテロメア機能不全について議論したい．

Regulation of Telomere Integrity by ATM Family Proteins
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Ⅰ．テロメア配列の維持機構

●1．テロメアDNAの維持に関与する“力”

1970年代に提唱されたように，線状のDNA末端は

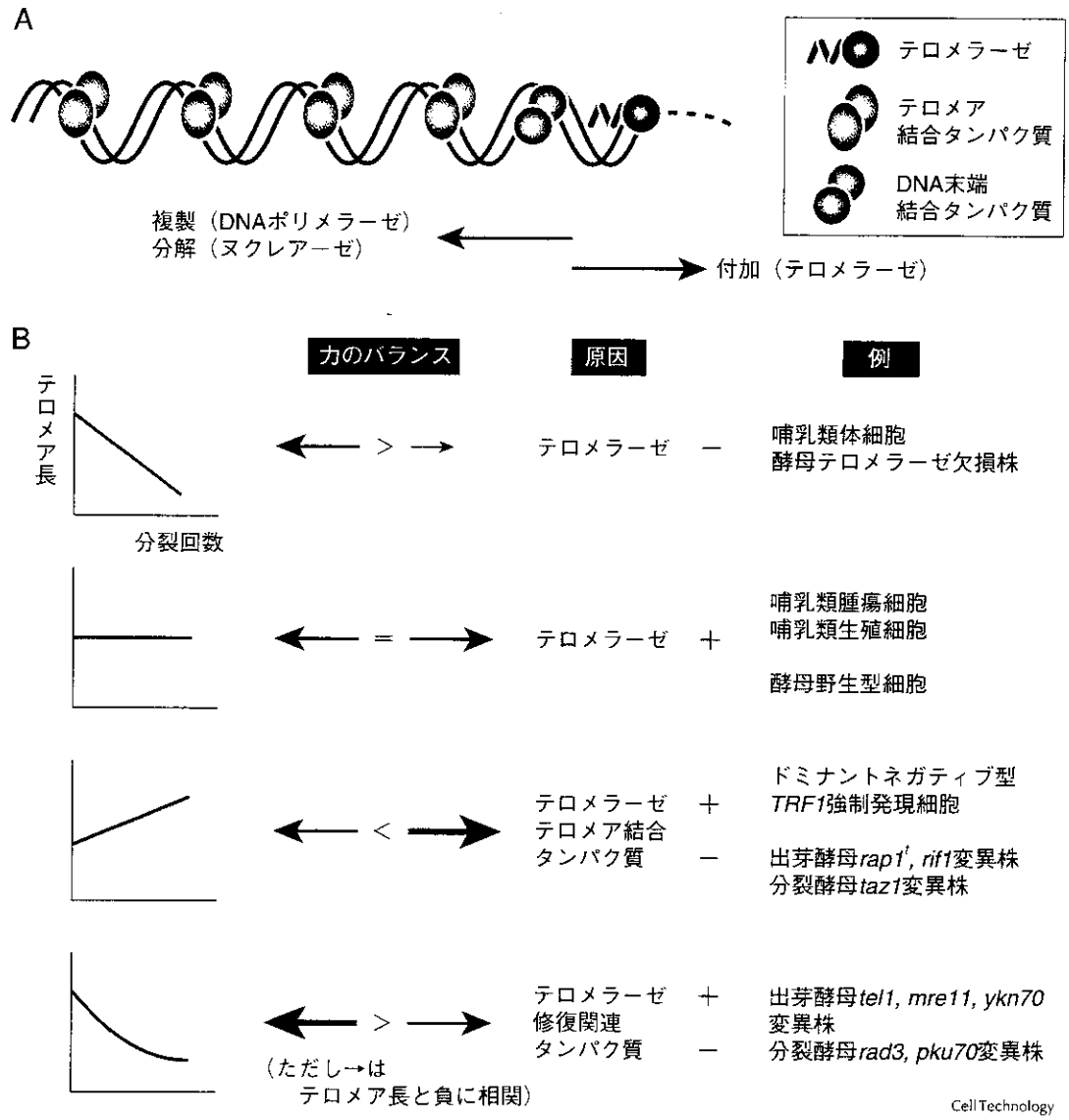


図1 ● テロメアDNAの制御機構

A：テロメアDNAに直接結合するタンパク質とテロメア長を制御する"力". テロメラーゼはテロメア反復配列の伸長反応を行い，DNA末端結合タンパク質Kuはテロメア DNAを分解から保護する機能を持つ．テロメア長は，テロメラーゼによる伸長，複製による短縮およびヌクレアーゼによる分解という3つの活性の均衡によって決定されている．

B：力の均衡変化によるテロメア長変化．

複製のたびに短くなる宿命を持つ．事実，分裂寿命がある培養細胞では，テロメアDNAは分裂を繰り返すたびに短縮していくことが観察された．一方，細胞がひとたび不死化すると，テロメアの短縮はほとんどの場合観察されなくなる．この，テロメア短縮の抑制にはテロメラーゼが重要な役割を担っている．この酵素の触媒サブユニットには逆転写酵素に見られるモチーフが存在し，内在するRNA成分を鋳型としてDNA末端にテロメア反復配列を付加する活性を有している．

ここで，テロメアDNAを維持する機構について考えてみよう（図1）．テロメア長の変化は，テロメアで起こる短縮反応と伸長反応との差し引きによって説明できる．哺乳類体細胞では，伸長反応をするテロメラーゼ活性が存在せず，テロメア長は複製ごとに短縮する方向に推移するが，酵母のような単細胞生物や，不死化した腫瘍細胞では短縮と伸長の間で均衡状態となり，一定の範囲内でテロメア長は維持される．

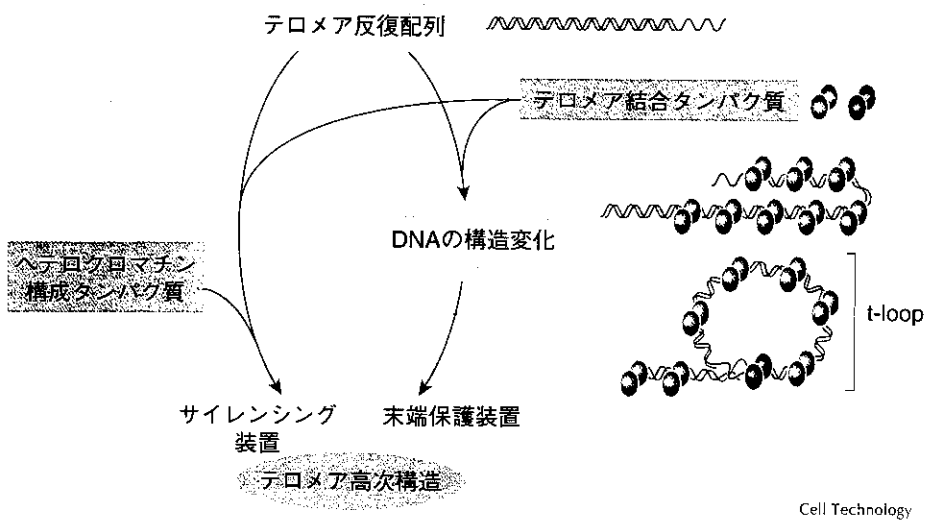


図2● テロメア高次構造構築の階層性

テロメアDNAは数塩基対の配列を単位とした反復配列からなり，最末端は一本鎖領域から成っている（赤）．テロメア配列結合タンパク質は，テロメアDNAの高次構造の形成に関与し，末端を保護する役割を担うと同時に，ヘテロクロマチンの構成タンパク質とともに，より高次のタンパク質複合体形成にも関与している．

●2．テロメア長ホメオスタシスとテロメア結合タンパク質によるカウンティングモデル

テロメアDNAは種特異的な長さを持ち，その長さは世代を超えて一定である．これは，生殖系列の細胞でテロメラーゼ活性が高いことと一見矛盾があるように見える．テロメラーゼ活性が高い細胞株においてもテロメア長は一定に保たれ，また新たに形成された短いテロメアは，もとから存在する他のテロメアと同程度の長さになるまで伸長し，安定化する現象が知られている．このことは，細胞にはテロメア反復配列の長さを個々の末端ごとに測定し調整する*cis*に働くメカニズムが存在していることを示している．出芽酵母を用いた実験により，末端に存在するテロメア結合タンパク質の数を一定に保つ機構の存在が示された[1]．つまり，テロメア配列の長さがそこに存在する結合タンパク質の数として測定されているわけである．

出芽酵母で人工的にテロメア長をコントロールする系を構築したMarcandらは，DNAポリメラーゼによる複製反応によるテロメアの短縮と，テロメラーゼ反応によるテロメア伸長との釣り合いのメカニズムを実験的に示した[2]．テロメラーゼ活性が存在しない条件下で，テロメアの短縮速度はその時のテロメアの長さに依存しない．一方，テロメア付加による伸長反応はテロメア長と負の相関を示す．この2つの力の釣り合いによりテロメアの平衡状態における長さが規定されていると考えられる．テロメア結合因子は，テロメアの長さに依存したテロメラーゼ活性の抑制に直接的に関わっていると考えられているが，その機構の詳細は今のところ明らかにされていない．1つの可能性は，タンパク質の結合によりDNAの構造変化が起こり，テロメラーゼの基質となりえないような構造をとるということである．実際，ヒト細胞においてテロメア末端がt-loopと呼ばれる特殊な構造をとっていることが示された[3]（図2）．その構造中では末端は隔離されており，外から酵素が作用しにくくなっている．t-loopの形成にはテロメアDNAに直接結合する活性を持つTRF1，TRF2が関与しているらしい．同様のループ構造がどの程度普遍的に存在するかについては今後の解析が待たれるが，DNA構造のなんらかの変化を介してテロメラーゼの末端へのアクセスを制御しているという基本的な図式は共通だろうと考えられている．

●3．テロメア長を制御する因子：出芽酵母を用いた解析から

出芽酵母を用いた遺伝学的解析から，テロメアDNAの維持に関わる数多くの因子が同定されている．それらのうち，テロメアの伸長を引き起こす変異は，そのすべてが表現型の発現にテロメラーゼを必要とする．このことは，テロメアDNAの伸長は主にテロメラーゼの作用に依存し，DNAポリメラーゼのslippageや反復配列間の組換えによるgene conversionは伸長の主要な経路ではないことを示している．

一方，テロメアの短縮を引き起こす変異は2種類

表1● 酵母のテロメア長制御因子の欠損がもたらす表現型

遺伝子	出芽酵母		分裂酵母	
	テロメア長	サイレンシング	テロメア長	サイレンシング
MEC1 *rad3*$^+$	わずかに短縮	減弱	短縮	わずかに減弱
TEL1 *tel1*$^+$	短縮	ほぼ正常	正常	?
MRE11 *rad32*$^+$	短縮	正常	正常	?
YKU70 *pku70*$^+$	短縮	減弱	短縮	わずかに減弱

酵母のテロメア長，テロメアでのサイレンシングの制御に関与する遺伝子に関し，欠損株の示す表現型を出芽酵母，分裂酵母で比較した．遺伝子の欄は上段が出芽酵母の遺伝子，下段が対応する分裂酵母の遺伝子である．

に分類することができる．*EST1*，*EST2*，*EST3*，*EST4*（別名*CDC13*），および*TLC1*は*in vivo*でのテロメラーゼ活性に必須な因子をコードしており，その欠損によるテロメアの短縮は，（もしテロメア喪失が細胞にとって害がないと仮定すれば）終わりがない（図1）．実際には，テロメアの喪失により細胞の生存率が低下し老化表現型を呈するようになるが，その後細胞集団の中から，テロメラーゼに依存せずテロメアを維持する新たな機構を獲得した細胞が選択的に増殖するようになる．他方，欠損により徐々にテロメアが短縮するものの，あるところで短縮が止まりその長さで新たな平衡状態となるようなものもある（図1）．このタイプのものには，後述する*TEL1*や，非相同組換えに働く因子をコードする*MRE11-RAD50-XRS2*，Kuヘテロ二量体を構成する*YKU70-YKU80*がある（表1）．組換え因子は，DNAの二重鎖切断部位において末端配列の保護およびDNA鎖特異的なプロセシングを行っていることが示されている．テロメアの主要な機能が染色体末端を損傷末端から区別することであるということからすると，組換え修復と同様な因子がテロメアDNAの維持に必要であるという事実は一見奇妙に感じられる．しかしながら，DNA合成期での複製の際に，テロメアの複合体構造が一部解消されることが考えられ，おそらく細胞周期の中の限られた期間内で起こるテロメア末端でのプロセシング，あるいはヌクレアーゼによる（受動的な）分解が，テロメア長を決定する因子となっているのであろう．これは，前述の変異株におけるテロメア長の変化には細胞周期の回転が必要であるという実験事実とよく合致する．

実際にテロメア末端で組換え因子が機能していることを示す証拠として，哺乳類Kuタンパク質の少なくとも一部は*in vivo*でテロメアに結合しているとの報告がある[4]．この局在化の意義，細胞周期における動態に関してはさらなる解析が必要であろう．

Ⅱ．テロメア複合体構造の維持とATMファミリータンパク質

●1．テロメアDNAとテロメア高次構造構築

テロメアの機能はDNAとそこに局在するタンパク質から成る複合体によって担われている（図2）．テロメア反復配列に直接結合する活性を持つタンパク質のうち，哺乳類TRF2，出芽酵母Cdc13pの活性喪失はテロメアを他の切断末端と区別する機能を失わせ，その結果細胞周期チェックポイント依存的な増殖停止が起こる[5),6)]．このことは，テロメアとしての最も基本的な機能である末端のキャッピングは，反復配列とそこに結合する最小限のタンパク質によって担われていることを示している．

一方，テロメアDNAは多数のタンパク質と複合体を形成することにより，ヘテロクロマチン様の構造となり，その結果近接した遺伝子の転写が抑制された状態となっている．この転写抑制（サイレンシング）に関与する因子が最近数多く単離されてきているが，それらの欠失によって増殖の停止は起こらない．したがって，ヘテロクロマチンの形成それ自体はテロメアの持つキャップ機能には必須でない．高

次構造の形成は，染色体が分化，老化などより高次な生命現象を制御するうえで重要な機能を果たしているものと考えられている．

●2．染色体不安定性症候群で見られるテロメア機能異常

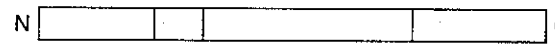
染色体が不安定となる一群の遺伝病が知られている．近年，それらの原因遺伝子が相次いで明らかにされ，遺伝子産物がDNA上の複製，修復過程に関与することが示された．これらのうち，毛細血管拡張性運動失調症（ataxia telangiectasia；AT）の原因遺伝子産物ATM（ataxia telangiectasia mutated）はプロテインキナーゼ活性を持ち，p53のリン酸化を制御することで細胞周期チェックポイントに関与する[7),8)]と同時に，癌抑制遺伝子の産物であるBRCA1をリン酸化し[9)]，c-ablを介してRAD51とRAD52の相互作用を制御する[10)]ことで，DNAの組換え修復に関与していることが示されている．*ATM*欠損細胞で起こることとして，染色体異常の頻発が知られているが，なかでも特徴的なのはテロメア末端どうしが融合した異常染色体の出現である[11)]．

*ATM*変異細胞中では，*in vitro*テロメラーゼ活性は正常であることが示されている[11)]．またテロメア長短縮の亢進はテロメラーゼの発現がない細胞のみで起こり，テロメラーゼの発現がある不死化した*ATM*欠損細胞では観察されないとの報告もある[12)]．興味深いことに，*ATM*欠損線維芽細胞に正常型*ATM*遺伝子を導入すると，異常染色体の出現頻度は減少するが，その際テロメアの長さには際立った変化が観察されない[13)]．つまり，テロメアの異常はテロメアの長さと直接的な関係がない．このことは，*ATM*欠損によるテロメアの機能異常の直接の原因は，テロメアDNAが短小化したことではなく，テロメアの持つなんらかの機能の低下であることを示唆している．

最近，Zhuらはテロメラーゼにテロメア配列を付加する以外の機能として末端をキャップする役割があることを提唱している[14)]．*in vitro*での伸長反応の際に，活性を持つ酵母テロメラーゼが反応後もDNAに結合し続けるとの報告もある[15)]．以上の結果は，ATMタンパク質がテロメアのキャップ構造の維持に関与し，その欠損がテロメラーゼの持つキャップ機能によって少なくとも部分的に肩代わりされるということで説明できる．

表2●ATMファミリータンパク質

	ATMオーソログ	ATRオーソログ
哺乳類	ATM	ATR
ハエ		Mei-41
線虫		Atl-1
出芽酵母	Tel1p	Mec1p
分裂酵母	Tel1	Rad3



ロイシンジッパー，プロリンリッチ領域など　PIキナーゼドメイン

多くの生物種で2種類の非常に相同性の高いタンパク質が発現している．ハエ，線虫ではこれまでのところATMオーソログは単離されていない．これらは総アミノ酸残基数3000までの巨大なタンパク質であり，いくつかの機能ドメインを有している．

●3．酵母ATMファミリータンパク質とテロメア

ATMファミリータンパク質は真核生物において広く保存されており，またほとんどの生物種において2種類の構造的に類似したタンパク質が存在する．そのうちの1つはATMと構造的に類似した分子であり，他方はATMのパラログであるATRとより類似した構造を持っている（表2）．これらのタンパク質はC末端に脂質リン酸化酵素のモチーフを持つが，実際には脂質に対してではなく，タンパク質キナーゼの活性を有している．

これらの分子の多くは，前述のように染色体DNAの損傷に応答して細胞周期の進行を停止させる機構（細胞周期チェックポイント制御系）に関与している．それに加え，これらの分子のうち出芽酵母*TEL1*，分裂酵母*rad3*$^+$遺伝子の欠損により，テロメア長の異常が観察される[16)]（表1）．すなわち，*rad3*変異株のテロメアDNAは野生型株に比べ短縮した状態で平衡状態となる．また，*rad3*，出芽酵母*mec1*の欠損株ではテロメア近傍でのサイレンシングの欠損も観察される[17),18)]．

哺乳類ATMとATRは細胞周期チェックポイント制御において重複した機能を持つことが示唆されているが，テロメアの制御においても，ATMオーソログとATRオーソログは重複した機能を持っている．

すなわち，分裂酵母の*tel1*$^+$と*rad3*$^+$，出芽酵母の*TEL1*と*MEC1*の二重破壊株では，テロメアは新たな平衡状態を獲得することなく短縮を続け，テロメア配列を喪失した染色体が生ずる[19),20)]．その結果，細胞の生存率は極端に低下するが，その後その欠損をバイパスする機構を獲得した細胞が出現し，増殖は部分的に回復する．

筆者らは最近分裂酵母の*rad3*$^+$遺伝子上の突然変異の検索を行い，テロメアに関する機能に関する温度感受性変異，*rad3-h4*を単離した．この変異株においては，許容温度では野生型と同様のテロメア長を示すものの，制限温度にシフトすると徐々にテロメアが短小化し，やがて*rad3*欠損細胞と同程度のテロメア長となって安定化する．いずれの温度においても，細胞周期チェックポイント機能は正常であることが特徴である．従来の遺伝学的解析からも，*rad3*欠損によるテロメアの短縮表現型は細胞周期チェックポイント機能の異常とは直接関係がないことが示唆されていた[17)]が，*rad3-h4*変異の単離によりそれが再確認されたことになる．

Rad3タンパク質が持つ，チェックポイント系とは独立した機能の詳細は今のところ明らかでないが，*ATM*関連遺伝子の欠損が様々な生物種において染色体異常，特に反復配列間の組換えの異常を誘発することが報告されることから，DNA損傷に対する修復系の活性の制御，複数の修復経路のうちどの系を選択するかの切替などに関与している可能性がある．事実，筆者らは*rad3*変異株で相同／非相同組換え経路の選択が野生型と異なることを見い出している．また，ニワトリ細胞株DT40を用いた遺伝学的解析によって，ATMが相同組換え経路に関与していることが明らかにされている[21)]．

●4．ATMはテロメア構造and/orテロメア局在を制御する？

ヒト*ATM*欠損細胞では，テロメアと核マトリックスとの相互作用が異常であり，またテロメア近傍でのヌクレオソームの周期性にも異常が見られるという報告がある[22)]．しかしながら，欠損細胞でテロメア結合タンパク質やテロメラーゼの挙動は野生型細胞と顕著な違いはない．このことは，ATMがテロメアDNAを制御しているというよりも，むしろテロメアのクロマチン構造を制御している可能性を示唆している．*mec1*, *rad3*変異株で見られるサイレンシングの異常は，酵母においてもATMファミリータンパク質がテロメアの構造を直接にしろ間接にしろ調節していることを示唆する．

ヘテロクロマチン構造を制御するタンパク質として，最近注目を集めているのがクロモドメインと呼ばれる領域を持つ一群のタンパク質である．これらのタンパク質としてショウジョウバエ，哺乳類のHP1や分裂酵母のSwi6などが知られている．Swi6はセントロメア，テロメア，接合型遺伝子座という分裂酵母の3つのヘテロクロマチン領域に局在が認められ，その欠損によりそれらの領域における転写抑制をほぼ完全に解除する．しかしながら，変異株のテロメアDNAの長さには変化がない．一方，*rad3*変異ではテロメアDNAの長さは大きく変化するが，転写抑制は部分的に解除されるだけである．このことから，*rad3*変異株においてテロメアヘテロクロマチン構造全体が根本から変化している可能性は少ないと考えられる．したがって，もし仮にRad3がクロマチン構造を制御しているとしても，その制御はテロメア末端近傍だけに留まるか，あるいはヘテロクロマチン構造自体ではなく，DNAと高次構造の間に存在する時間的あるいは空間的な“中間”ないし“下部”構造を調節しているのであろう．

ATMファミリータンパク質がクロマチンの高次構造を制御する機構に関しては，ATM，ATRとヒストンデアセチラーゼHDAC1，HDAC2の直接相互作用に関する報告がある[23),24)]が，この相互作用の意味するところについては今のところ明らかになっていない．

■Ⅲ．DNA修復と複合体形成と…

*ATM*ファミリー遺伝子の欠損によるテロメア表現型を，DNAおよび高次構造の面から議論してきた．現時点では，ATMファミリータンパク質がDNA修復系などを直接制御することでテロメア長維持の力のバランスを変えている可能性，テロメアの高次構造を変化させることで間接的にテロメア長を変化させている可能性，あるいはその両方を制御している可能性の3つを考慮する必要があろう（図3）．

出芽酵母のMec1がDNA損傷後のKuタンパク質

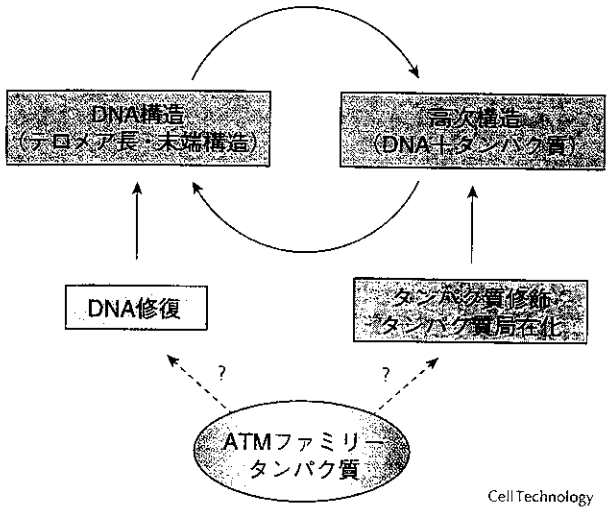


図3● DNAが先か、高次構造が先か？
ATMファミリータンパク質がテロメアのDNAを直接制御する可能性，テロメア近傍のクロマチン構造の制御を介して間接的にテロメアDNAを制御している可能性がある．

などの局在化を制御していること[25),26)]から，ATMファミリー遺伝子産物が組換え関連因子を介してテロメアの機能を制御していることが考えられる．しかし，テロメア機能の異常による2つの表現型（テロメア長の短縮，サイレンシングの減弱）をそれぞれの変異株間で比較してみると，*ATM*欠損と組換え因子の欠損とは，表現型が必ずしも一致せず，*ATM*ファミリー遺伝子が何らかの因子のテロメアへの局在，あるいはタンパク質の活性を制御しているというような単純なメカニズムではすべての現象を説明できない．

一方，ATMタンパク質がDNA末端に直接結合する能力を有しているとの報告がある[27),28)]．*in vivo*でATMファミリータンパク質がテロメアと直接結合している可能性について今後の検討が必要であるが，この結果は，ATMが調節的な役割だけではなく，構造的な役割も持っている可能性を示している．この点，示唆的なのは最近ATMファミリーと構造的に似たドメインを持つタンパク質TRRAPがヒストンH4アミノ末端のアセチル基転位酵素と転写のアクティベーターとをつなぐ因子として機能しているという報告である[29)]．TRRAPが1MDaにも及ぶタンパク質複合体の一部として遺伝子発現に関与していることを考えると，ATMファミリータンパク質が同様な複合体中で機能を果たしている可能性も検討する必要があろう．

いずれにせよ，ATMファミリータンパク質の持つ多面的な機能，すなわち細胞周期のチェックポイント，DNA二重鎖切断修復，クロマチンの構造変換が同じ分子によって統一的に制御されているということは非常に興味深い．1つの可能性は，一見独立な現象であるかのように考えられていた反応が実は生体内では有機的なつながりをもっているかもしれないということである．事実，DNA損傷によるクロマチンアセンブリーの誘導[30)]，クロマチンアセンブリー複合体によるDNA修復の制御[31)]など，クロマチンの変化と細胞周期チェックポイント，DNA修復の直接の関係を示唆する報告が散見されるようになってきている．酵母ではテロメア領域はDNA合成期の最も遅い時期に複製が行われるが，この複製時期の決定にもATRオーソログのMec1pは関与する[32),33)]．また，複製時期の決定がテロメアのクロマチン構造に依存しているという報告もある[34)]．

おわりに

ショウジョウバエのテロメアにはテロメラーゼによる付加配列が存在しないが，染色体末端は損傷末端と明らかに区別されている[35)]．したがって，一度テロメアであるということを規定する構造ができてしまえば，配列に依存せずテロメア構造を維持できる場合があるうるということになる．

しかしながら，テロメア機能の不全とテロメア長の短縮の間に相関があるのは明らかである．染色体の最末端に存在するという位置的な問題，G-C対が多いという配列的な問題がテロメア複合体をより不安定に，また損傷に対してより高感受性にしている．真核細胞は，DNAに作用する因子を総動員してテロメア構造を維持し，ゲノム情報をやっとの思いで保っているような印象を受けている．

そのような思いまでして守りたい染色体の線状性とは何なのか？どのような生命現象に必要なのか？少なくとも減数分裂の正常な進行には染色体が線状であることが必須である[19)]．発生，分化，老化などの高次生命現象と線状の染色体構造とはなんらかの関係があるのだろうか？その解答が待ち焦がれる．

追記 テロメアDNAの*in vivo*伸張は細胞周期中の限られた期間でのみ起こること[36),37)]，その反応にはDNAポリメラーゼα，δが必要であること[37)]が出芽酵母を使った遺伝学的な解析から示された．テロメアの高次構造が複製反応が進行している時期にのみ解消されることを示唆するデータである．また，ヒトATMタンパク質がBASC（BRCA1-associated genome surveillance complex）と名付けられたタンパク質複合体に含まれていることが最近明らかにされた[38)]．

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Identification of DNA *cis* Elements Essential for Expansion of Ribosomal DNA Repeats in *Saccharomyces cerevisiae*

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Saccharomyces cerevisiae **carries ~150 ribosomal DNA (rDNA) copies in tandem repeats. Each repeat consists of the 35S rRNA gene, the NTS1 spacer, the 5S rRNA gene, and the NTS2 spacer. The *FOB1* gene was previously shown to be required for replication fork block (RFB) activity at the RFB site in NTS1, for recombination hot spot (*HOT1*) activity, and for rDNA repeat expansion and contraction. We have constructed a strain in which the majority of rDNA repeats are deleted, leaving two copies of rDNA covering the 5S-NTS2-35S region and a single intact NTS1, and whose growth is supported by a helper plasmid carrying, in addition to the 5S rRNA gene, the 35S rRNA coding region fused to the *GAL7* promoter. This strain carries a *fob1* mutation, and an extensive expansion of chromosomal rDNA repeats was demonstrated by introducing the missing *FOB1* gene by transformation. Mutational analysis using this system showed that not only the RFB site but also the adjacent ~400-bp region in NTS1 (together called the EXP region) are required for the *FOB1*-dependent repeat expansion. This ~400-bp DNA element is not required for the RFB activity or the *HOT1* activity and therefore defines a function unique to rDNA repeat expansion (and presumably contraction) separate from *HOT1* and RFB activities.**

In most eukaryotic organisms, the ribosomal RNA genes (rDNA) are present in long tandem repeats at one or a few chromosomal loci, the nucleolar organizers, and function in the synthesis of rRNA. In the yeast *Saccharomyces cerevisiae*, approximately 150 rDNA tandem repeats are located on chromosome XII. A single unit of rDNA consists of two transcribed genes (5S and 35S rRNA genes) and two nontranscribed regions (NTS1 and NTS2) (Fig. 1). The 35S rRNA gene is transcribed by RNA polymerase I (Pol I), yielding the 35S rRNA, which is then processed into mature 18S, 5.8S, and 25S rRNAs, while the 5S rRNA gene is transcribed by Pol III. Two DNA elements related to DNA replication, the origin of replication (*ARS*) and the replication fork barrier (RFB), are located in NTS2 and NTS1, respectively. During each round of DNA replication, a bidirectional replication is initiated at, on the average, one in five *ARS* sites (2, 21). The RFB located near the end of the 35S rRNA gene allows the progression of the replication fork in the direction of 35S rRNA transcription but not in the opposite direction (2, 3, 19). The RFB site overlaps the E element of *HOT1* (17, 35). (Actually, two closely spaced sites, RFB1 and RFB2, are present in this region [37], but we call these sites collectively the RFB site in this paper.) *HOT1* was originally discovered as a DNA element that stimulates genetic exchanges at nearby regions when inserted at a non-rDNA site (17). Two elements were subsequently identified as essential for *HOT1* activity: the I element, which corresponds to the Pol I promoter region, and the E element, which overlaps the enhancer for Pol I transcription originally identified by Elion and Warner (6). Thus, *HOT1* activity appears to be causally related to stimulation of transcription by Pol I.

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The total number of rDNA repeats per genome varies greatly depending on the organism. For a given organism, the repeat number appears to be maintained at an appropriate level, e.g., approximately 150 per haploid genome for *S. cerevisiae*. However, variations of the repeat numbers were observed quite often, and most organisms appear to have the ability to alter repeat numbers in response to changes in intra- as well as extracellular conditions. For example, we have previously shown that the absence of an essential subunit of Pol I triggers a gradual decrease in the number of rDNA repeats to about half the normal level and reintroduction of the missing Pol I gene induces a gradual increase of the number of repeats back to the original level (18). By analogy to this observation, one can imagine that a harmful deletion of a significant fraction of rDNA repeats by homologous recombination could be repaired by the ability of cells to expand repeat numbers, as was in fact observed for *Drosophila bobbed* mutations (26, 34). In addition, it was recently discovered that yeast mutants defective in the Pol I transcription factor UAF give rise to variants that are able to grow by transcribing chromosomal rDNA repeats by Pol II and that the switch to growth using the Pol II system is accompanied by a large expansion of rDNA repeats up to approximately 400 (25, 36). In this case, the repeat expansion clearly represents an adaptation process to growth without the intact Pol I system. Thus, although an extensive recombination activity in rDNA repeats may be harmful to cells, as discussed in connection with cell aging and *SIR2*-dependent gene silencing in yeast cells (5, 11, 29), some limited and regulated recombinational activities within rDNA repeats appear to be important for cellular adaptation and repeat number maintenance, in addition to their well-discussed role in the maintenance of sequence homogeneity among many rRNA genes. However, although extensive studies were carried out on the mechanism of recombination within rDNA repeats in *Drosophila* and yeast and some specific models were proposed

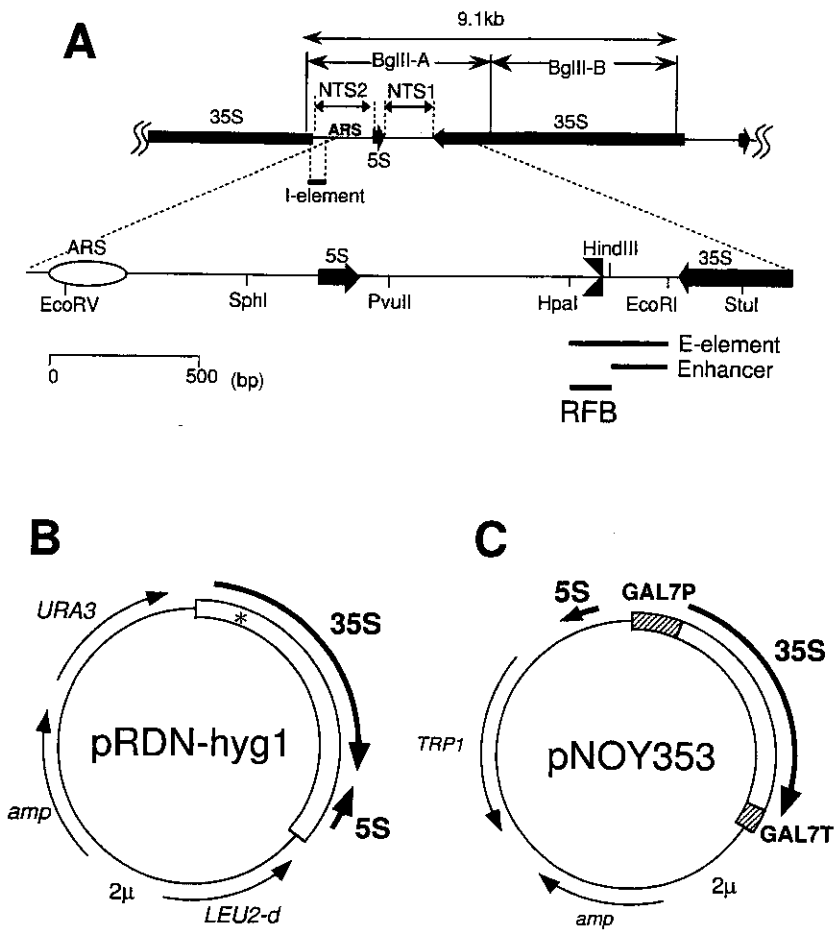

FIG. 1. (A) Structure of rDNA repeats in *S. cerevisiae*. The locations of the 35S and 5S rRNA genes (with the direction of transcription indicated by arrows), the two nontranscribed spacer regions (NTS1 and NTS2), *ARS* (replication origin), and the *HOT1* I-element are shown in the upper part. *Bgl*II A and B DNA fragments are also shown. NTS1 and its surrounding regions are expanded. Three solid bars represent the *HOT1* E-element, Pol I Enhancer, and RFB (the replication fork blocking site, also indicated by ❳). (B) Structure of pRDN-hyg1 (4). This plasmid carries a single copy of rDNA repeats obtained by cutting the repeats with *Sma*I (hence the copy starting from −206 and ending at −207; the numbering is with respect to the Pol I transcription start site as +1). There is a mutation in the 18S rRNA gene (indicated as an asterisk) which makes the ribosome hygromycin B resistant. (C) Structure of pNOY353. This plasmid carries the 7.5-kb *Bam*HI-*Xho*I fragment, which contains *GAL7*-35S rDNA (the 35S rRNA coding region fused to the *GAL7* promoter as described by Nogi et al. [23]) inserted between the *Bam*HI and *Sal*I sites of the pTV3 vector (27). This plasmid also contains the 1,085-bp *Pvu*II-*Eco*RV fragment carrying the 5S rRNA gene (see panel A) inserted in the *Sma*I site upstream of the *GAL7* promoter. The 35S rRNA coding region contains up to the *Hin*dIII site, +6935. Thus, the Pol I enhancer is present but the region from *Hin*dIII to *Pvu*II in NTS1 and the Pol I promoter region (from −1 to the *Eco*RV site at +8757 or the 381-bp region) are absent.

(7, 18, 33, 39; for studies on *Drosophila*, see the review in reference 12), actual molecular mechanisms unique to rDNA have remained largely unknown.

An important gene required for rDNA repeat expansion and contraction discovered in the yeast system is *FOB1* (18). *FOB1* was originally identified as the gene required for both replication fork-blocking activity (RFB activity) at the RFB site within the rDNA repeats and *HOT1* activity in a recombination test system outside the rDNA repeats (20). Using the Pol I-dependent rDNA repeat expansion-contraction assay system mentioned above, it was subsequently demonstrated that *FOB1* is required for efficient rDNA expansion and contraction (18). In addition, mutation in the *FOB1* gene was found to reduce the frequency of the formation of extrachromosomal rDNA circles from the rDNA repeats (5) as well as the frequency of actual recombination as assayed by the use of a marker gene integrated within rDNA repeats (K. Johzuka and T. Horiuchi, unpublished experiments). Because *FOB1*-dependent replication fork blocking takes place at the RFB site (3, 19) and because pausing of replication is known, at least in bacterial systems, to stimulate both DNA double-strand breakage (22) and recombination (13, 14, 28), we have previously proposed that *FOB1*-dependent rDNA repeat expansion and contraction takes place as a result of *FOB1*-dependent replication fork blocking at the RFB site, presumably involving double-strand breakage and repair of the break via gene conversion, as illustrated in Fig. 2 (see the legend for further explanation). If this proposal is correct, that is, if the *FOB1*-dependent replication fork block is in fact the cause of the stimulation of rDNA repeat expansion and contraction by *FOB1*, the RFB site located near the end of the 35S rRNA gene should be essential for this expansion and contraction process. To examine this

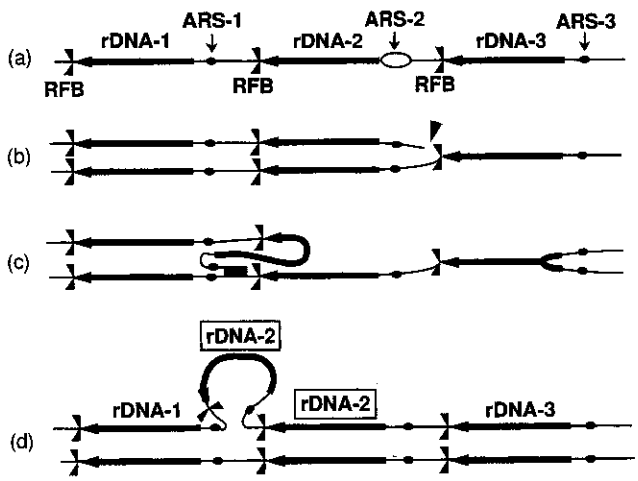

FIG. 2. The fork block-dependent recombination model for rDNA repeat expansion and contraction. The positions of *ARS* and RFB are shown as solid dots and], respectively. Individual lines represent chromatids with double-stranded DNA. In this model, DNA replication starts from one of the *ARS* sites (ARS-2) bidirectionally (a). In the yeast rDNA repeats, about one in five *ARS* sites is used as an active origin (2, 21). A rightward replication fork is arrested at the RFB site, and this arrest is supposed to stimulate a double-strand break of DNA at a nearby site (indicated by an arrowhead in row b). A strand invasion at a homologous duplex (a downstream sister chromatid near ARS-1 in this example) takes place (c), and a new replication fork is formed. The new replication fork meets with the leftward replication fork from the upstream site, resulting in formation of two sister chromatids, one of which gains an extra copy of rDNA, indicated as boxed rDNA-2 (d). If the strand invasion is at a site in a upstream repeat (e.g., near ARS-3), a loss, rather than a gain, of an rDNA repeat is expected. This model was proposed previously to explain the observed strong dependence of rDNA repeat expansion and contraction on *FOB1* (18).

question and to find whether any other DNA *cis* elements surrounding the RFB site are required for repeat expansion and contraction, we have developed a system in which these questions can be studied by mutational analysis. Obviously, the presence of redundant rDNA copies makes the mutational analysis very difficult. We have constructed a yeast strain in which the majority of rDNA repeats are deleted, leaving two copies of rDNA covering the 5S-NTS2-35S regions and a single intact NTS1 region in between and whose growth is supported by a multicopy helper plasmid which does not carry the intact NTS1. Using this strain, initial mutational analyses were carried out. We have found that the RFB site is in fact essential for *FOB1*-dependent rDNA repeat expansion. We have also found that in addition to the RFB region, the adjacent ~400-bp region in NTS1 is required for the efficient repeat expansion but the Pol I transcription enhancer region is apparently not required. The ~530-bp region, which combines the RFB region with the newly identified ~400-bp region, is now called EXP (for expansion of rDNA repeats). The requirement of the new DNA *cis* element(s) independent of the RFB site can now define a new function(s) which is involved in the rDNA repeat expansion independent of the RFB activity.

MATERIALS AND METHODS

Media, strains, and plasmids. SD is a synthetic glucose medium (16). SGal is the same as SD except that 2% glucose is replaced by 2% galactose. Both SD and SGal were supplemented appropriately with amino acids and bases to satisfy nutritional requirements and also to retain unstable plasmids (16).

Yeast strains and plasmids used in this study are listed in Table 1. Disruption of *FOB1* was described previously (18). Plasmid pTAK101 was constructed by inserting the *FOB1* gene amplified by PCR (20) into the *Bam*HI site of YEplac181 (8). TAK200 was constructed from NOY408-1b as previously described (4, 24) and is described in Results.

NTS1 mutants A to G were constructed from TAK201 by gene replacement (16). The region (~1.1 kb) covering the NTS1 and the 5S RNA gene was subdivided into seven segments, A to G (see Fig. 3 and below), and each segment was replaced individually with the 1,162-bp *Hin*dIII fragment containing *URA3* as follows. Two DNA sequences of approximately 500 bp that flank a segment to be replaced were amplified by PCR using DNA prepared from TAK201. Each of the primers used for PCR had recognition sites at the 5′ ends, one for *Bam*HI (distal primer) and the other for *Pin*AI (proximal primer, i.e., the primer containing the site to be used for connection to the *URA3* fragment). The two PCR products were digested with these two enzymes and cloned together into the pUC18 vector at the *Bam*HI site. A DNA fragment consisting of the 1,162-bp *Hin*dIII fragment containing *URA3* and additional *Pin*AI sites at both ends was constructed by PCR, cleaved with *Pin*AI, and inserted at the *Pin*AI site between the two 500-bp flanking sequences in pUC18 in the orientation that would make *URA3* and the 5S rRNA gene face the same direction. The resultant recombinant plasmid was digested with *Bam*HI. The fragment containing *URA3* and the two flanking sequences was separated from the vector portion and then introduced into TAK201 by transformation for replacement of the pertinent segment with *URA3*. PCR was used to confirm the positions and the size of the insert expected from the correct replacement. The positions of the segments replaced by *URA3* are as follows (using the conventional rDNA repeat numbering system, starting with +1 at the site of the start of Pol I transcription and increasing in the direction of 35S rRNA transcription): G, 6750 to 6934; F, 6935 to 7063; E, 7064 to 7193; D, 7209 to 7326; C, 7327 to 7462; B, 7463 to 7712; and A, 7713 to 7895. (A gap of 15 bp is present between the E and D segments but is irrelevant to the experimental design and the conclusion.)

Determination of the copy number of rDNA repeats. In the rDNA repeat expansion experiments, the number of rDNA repeats was determined after ~45 generations of growth. The number of generations was estimated based on the observation that a single colony with a diameter of 1 mm contained ~2×10^5 cells and the consequent assumption that cells in colonies of this size corre-

TABLE 1. Yeast strains and plasmids used in this study

Designation	Genotype and comments	Reference
Strains		
NOY408-1b	*MAT*a *ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100* pNOY102	23
TAK200	Same as NOY408-1b except for deletion of rDNA repeats, leaving two copies (Fig. 3), and for the presence of pRDN-hyg1 instead of pNOY102	
TAK201	Same as TAK200 except for the presence of *fob1*Δ::*HIS3*, and pNOY353 instead of pRDN-hyg1	
Plasmids		
pRDN-hyg1	Multicopy plasmid carrying *rdn-hyg1*, *rdn-ani1*, *leu2-d*, *URA3*, 2 μm, and Amp^r	4
pNOY353	Multicopy plasmid carrying *GAL7*-35S rDNA, 5S rDNA, *TRP1*, 2 μm, and Amp^r	24
YEplac181	Multicopy plasmid vector carrying *LEU2* and 2 μm	8
pTAK101	YEplac181 carrying *FOB1*	

sponded to progeny 18 generations from the individual ancestor cells. The *FOB1* gene was introduced into the control strain (TAK201) and NTS1 substitution mutants A to G by transformation using pTAK101. Colonies 1 mm in diameter were picked from Leu$^+$ selection plates and restreaked on the same plates, and the same-sized colonies were taken to inoculate the supplemented SGal medium. Cells were then grown for nine generations before being harvested, thus making a total of ~45 generations after the introduction of the *FOB1* gene. Control transformation was done using the vector plasmid YEplac181, and Leu$^+$ transformants ("vector transformants") were subjected to the same processes. DNA was then isolated, digested with *Bgl*II, subjected to agarose gel electrophoresis (1% agarose), and analyzed by Southern hybridization using probes for rDNA (probe 2 for mutants A to C and probe 1 for D to G [see Fig. 5C]) and for *MCM2* (a 1.4-kb fragment prepared by PCR) as described previously (30). Ratios of rDNA to *MCM2* were quantified, and the rDNA copy numbers were calculated by comparing these ratios (rDNA/*MCM2*) with the corresponding ratio obtained for TAK201, which contained two copies of rDNA. The amounts of radioactive probes hybridized were determined by phosphorimager analysis (BAS2000; Fujifilm).

Other methods. Samples for contour-clamped homogeneous electric field (CHEF) electrophoresis were prepared as described previously (31). Electrophoresis was carried out in a 0.8% agarose gel with 0.5× Tris-borate-EDTA (TBE) buffer, using CHEF-DRII (Bio-Rad, Richmond, Calif.) with a pulse time of 300 to 900 s and 100 cV for 68 h at 14°C. For the experiment in Fig. 4, a 1% agarose gel was used and the conditions for electrophoresis were altered to a pulse time of 60 to 120 s, 200 cV, and 40 h at 14°C. The gel was then stained with 1 μg of ethidium bromide (EtBr) per ml for 30 min at room temperature, photographed, and then subjected to Southern hybridization analysis (30). RFB activity was analyzed using two-dimensional (2D) gel electrophoresis as described previously (1). For field inversion gel electrophoresis, samples were prepared as previously described (31), digested with *Bam*HI, and subjected to gel electrophoresis in a 1% agarose gel with 0.5× TBE buffer, using FIGE Mapper (Bio-Rad). The conditions used included a switch time ramp of 0.4 to 2.0 s (linear shape), 180 cV (forward), 120 cV (reverse), and 20 h at 14°C. The gel was then stained with 1 μg of EtBr per ml for 30 min at room temperature, photographed, and subjected to Southern hybridization analysis (30).

RESULTS

Construction of a strain with two rDNA repeats. Most of the yeast rDNA repeats can be deleted using a method described by Chernoff et al. (4). Plasmid pRDN-hyg1 carries a single rDNA repeat with a recessive hygromycin resistance mutation in the 18S rRNA gene (Fig. 1B). This plasmid was first introduced into a control strain, NOY408-1b, using *URA3* for selection. The resultant strain was then subjected to a hygromycin resistant selection. Because the wild-type allele is dominant to the mutant *hyg1* allele, hygromycin-resistant mutants selected in this way are expected to have lost most of the chromosomal rDNA repeats by recombinational events. Because the rDNA repeats (~150 copies of the 9.1-kb repeat or ~1.4 Mb) represent a large fraction of the total length of chromosome XII (1.05 Mb of non-rDNA regions plus 1.4 Mb rDNA repeats, or ~2.5 Mb), degrees of reduction in rDNA repeat numbers can be assessed by measuring the length of chromosome XII in these hygromycin-resistant mutants by CHEF electrophoresis. Eight mutants were analyzed in this way, and the result is shown in Fig. 3A. Compared to the control strain (lane WT), a large reduction in the length of chromosome XII was evident for all the mutants analyzed and the remaining rDNA repeat numbers were estimated to be less than 20 in these mutants. We selected mutants 7 and 8 (Fig. 3A, lanes 7 and 8) and determined the copy numbers of their chromosomal rDNA repeats more precisely. Field inversion gel electrophoresis was carried out after digestion of their chromosomal DNA with *Bam*HI. As shown in Fig. 3B (lane 8), mutant 8 showed a band of approximately 57 kb. Knowing the DNA sequences of non-rDNA flanking the rDNA repeats, including the *Bam*HI sites closest to the rDNA, we can calculate that this value matches that for the presence of two rDNA copies, as shown in Fig. 3C. Mutant 7 failed to show any significant signal (Fig. 3B, lane 7) and may have lost the rDNA repeats completely. No further analysis was done on this mutant.

We selected mutant 8 (TAK200) for subsequent studies of rDNA repeat expansion. It should be noted that the end of the intact rDNA repeats at the right border (telomere proximal) is near the end of the 5S rRNA gene and that their left end is within the RFB region according to the GenBank sequence information (9). We determined the sequences around these two boundaries on DNA isolated from strain TAK200 and confirmed that they are identical to those in the data bank. Thus, TAK200 contains two intact 35S rRNA genes, two intact NTS2 regions, and a single intact NTS1 region (Fig. 3C). We note that the portion of the RFB region remaining at the left border is not sufficient to cause replication fork blocking, as judged from the results of previous experiments (19), leaving the single intact RFB in the middle for the RFB function in this strain. To prevent *FOB1*-dependent repeat expansion, thus stabilizing the two-copy state, the *FOB1* gene of TAK200 was disrupted by replacement with *HIS3*. In addition, plasmid pRDN-hyg1 was replaced by pNOY353. This plasmid contains the 35S rDNA fused to the *GAL7* promoter and, in addition, the 1.1-kb *Pvu*II-*Eco*RV fragment carrying the 5S rRNA gene and lacks most of NTS1 (i.e., the segment between *Hin*dIII and *Pvu*II which includes the RFB site [see the legend to Fig. 1]). This helper plasmid was used to minimize repairs of mutations to be introduced in the chromosomal NTS1 by gene conversion, which might take place when a single intact rDNA repeat is present on a helper plasmid like pRDN-hyg1. The resulting *fob1*-disrupted strain carrying pNOY353 (TAK201) was used for mutational analysis of rDNA repeat expansion. As expected from the limited number (two copies) of the chromosomal rDNA repeats and the *GAL7*-dependent rRNA synthesis through the helper plasmid, growth of TAK201 was galactose dependent.

Mutational analysis of the NTS1 region to identify *cis* elements required for rDNA repeat expansion. A systematic mutational analysis of the NTS1 region was done by dividing this region (and the 5S rRNA coding region) into seven segments (A to G) (Fig. 3C) (see Materials and Methods) and replacing each of them with the *URA3* gene, creating seven NTS1 substitution mutants (mutants A to G). Segment F corresponds to the 129-bp *Hin*dIII-*Hpa*I region which contains the RFB site. Segment G roughly corresponds to the 190-bp *Eco*RI-*Hin*dIII region which was originally defined as the Pol I transcription enhancer element (6). The *URA3* gene was placed in the same direction as the 5S rRNA gene. Expected mutational alterations in these mutant strains were confirmed by digestion of their DNA with *Bam*HI followed by Southern analysis, which showed no increase of rDNA repeat numbers, and by PCR analysis, which showed correct replacements of each segment with *URA3* (data not shown).

The seven NTS1 mutant strains, A to G, as well as the original control strain, TAK201, were transformed with a plasmid, pTAK101, which carries the wild-type *FOB1* gene, to induce a *FOB1*-dependent expansion of rDNA repeats (18). Transformants were selected using *LEU2* on the plasmid on

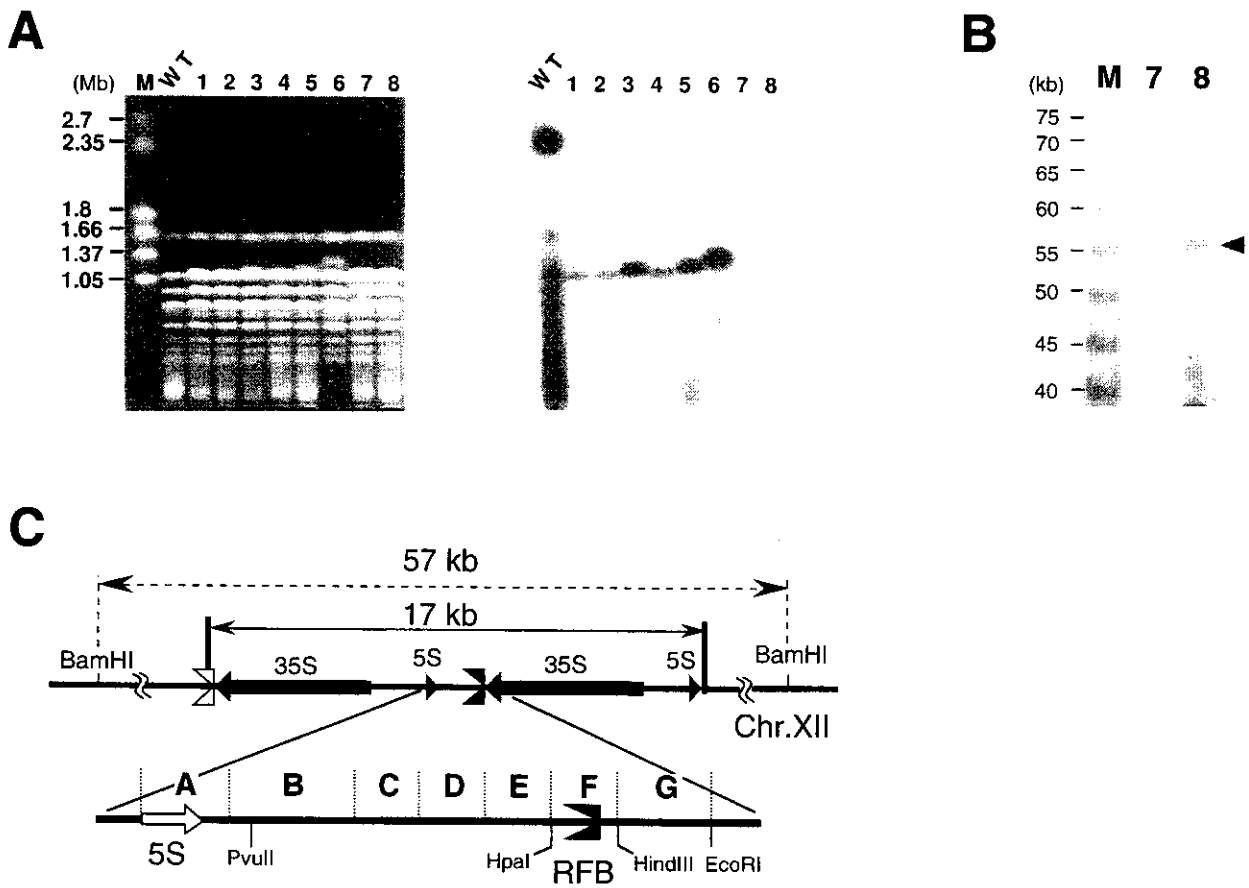

FIG. 3. (A) Analysis of the size of chromosome XII by CHEF electrophoresis. Eight independent hygromycin-resistant mutants (lanes 1 to 8), as well as the control strain, NOY408-1b, (lane WT), were examined. The left panel shows chromosome patterns revealed by staining with EtBr. The right panel shows an autoradiogram obtained after hybridization with an rDNA probe (probe 3 in Fig. 5C). Size markers (lane M) are made up of *Hansenula wingei* chromosomes (Bio-Rad). (B) Analysis of the sizes of rDNA repeats by field inversion gel electrophoresis. DNA samples prepared from mutants 7 and 8 (those shown in lanes 7 and 8 in panel A, respectively) were digested with *Bam*HI, subjected to the electrophoresis, and analyzed by hybridization using an rDNA probe (probe 3 in Fig. 5C). A band seen in lane 8 which corresponds to the size expected from two copies of rDNA is indicated by an arrowhead. Lane M is the 5-kb ladder provided by Bio-Rad. Because the amounts of the marker DNA molecules were much larger than the amount of fragment containing two copies of rDNA, nonspecific hybridization of the probe to the markers took place, providing positions of the markers conveniently on the same autoradiogram. (C) Structures of two rDNA repeats remaining in strain TAK201 and the NTS1-5S region subjected to the mutational analysis (expanded below). Seven segments, A to G, replaced by *URA3* individually in mutants A to G are indicated. The precise positions of each segment are given in Materials and Methods.

supplemented SGal plates which did not contain tryptophan or leucine. Five independent transformants were picked for each strain and purified by streaking on the SGal plates. Single colonies were then inoculated in liquid SGal medium with the same supplements, and the cells were grown for 18 h. Including colony formation twice on the plates and the following growth in the liquid medium, it was estimated that approximately 45 generations had occurred since the *FOB1* gene was introduced into these strains (see Materials and Methods). The size of chromosome XII was then analyzed by CHEF electrophoresis. The gels were stained with EtBr (Fig. 4A) and subjected to hybridization with an rDNA-specific probe and autoradiography (Fig. 4B). In the original strain (TAK201), which had two copies of rDNA, the band of chromosome XII overlapped those of chromosomes VII and XV in the EtBr-stained gel (Fig. 4A and B, lane 2-copies). This result was expected because the calculated size of chromosome XII in the original strain is 1.05 Mb, which is similar to the size (1.09 Mb) of chromosomes VII and XV. In contrast, chromosome XII in five transformants derived from the control strain (TAK201), which grew for 45 generations after introduction of the *FOB1* gene, was much larger than that of the two-copy control, and this was the case for all five independent transformants, as can be seen from the autoradiogram in Fig. 4B (lanes Control, FOB1). Each sample appears to represent a heterogeneous mixture of cells carrying chromosomes XII with different sizes, displaying smears rather than defined bands. For this reason, no defined bands corresponding to chromosome XII were observed for these samples on the EtBr-stained gel (Fig. 4A). The observed extensive expansion of rDNA repeats in these transformants requires the presence of the *FOB1* gene. No such expansion was observed for the five control cultures derived from five independent Leu^+ transformants, which were formed on introduction of the vector DNA without *FOB1* (Fig. 4A and B, lanes control, Vector). However, some small increases in the length of chromosome XII were clearly seen

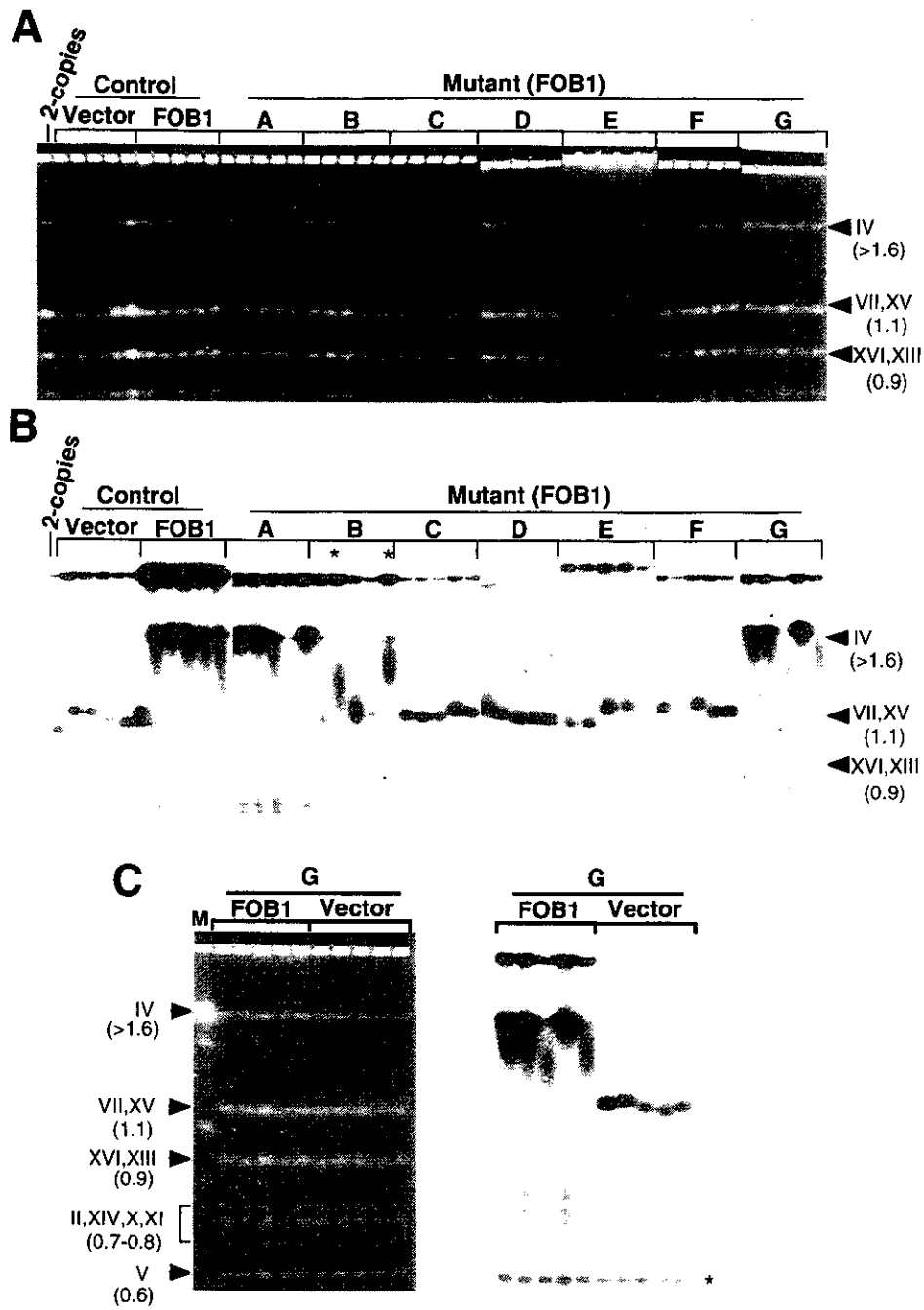

FIG. 4. Analysis of the size of chromosome XII in *FOB1* transformants of NTS1 substitution mutants by CHEF electrophoresis. (A and B) Five independent *FOB1* transformants derived from each of mutants A to G and from the control strain (TAK201) were analyzed along with five vector transformants of the control strain after ~45 generations. The reference TAK201, which had two rDNA repeats without expansion, was also analyzed (lane 2-copies). (A) Chromosomal patterns revealed by staining with EtBr; (B) autoradiograms obtained after hybridization with an rRNA probe (probe 3 in Fig. 5C). (C) Analysis of five *FOB1* transformants and five vector transformants derived from mutant G by hybridization using a *URA3* probe. The left panel shows chromosomal patterns revealed by staining with EtBr. The right panel shows an autoradiogram obtained after hybridization with the *URA3* probe. The position of chromosome V carrying the native *URA3* gene is indicated by an asterisk. On the right sides of the gels in panels A and B and on the left side in panel C, the positions of chromosomes and their sizes (in megabases) are indicated.

relative to the original two-copy rDNA strain, and the extents of the increases varied depending on the transformants obtained independently. The bands of chromosome XII were relatively homogeneous in sizes and could be seen even in the EtBr-stained gel. The observed *FOB1*-independent increase in rDNA repeat numbers is discussed below.

The effects of substitution mutations (A to G) on *FOB*-dependent rDNA repeat expansion were examined in the same

way, that is, by introducing the *FOB1* gene by transformation and analyzing the size of chromosome XII after ~45 generations of growth. Five independent *FOB1* transformants were analyzed for each mutant, together with five independent vector transformants. The results for the *FOB1* transformants are shown in Fig. 4A and B. The results for the vector transformants are not shown except for those derived from mutant G (Fig. 4C; see below), but all the vector transformants showed only a limited increase in the size of chromosome XII, as with the vector transformants derived from TAK201 mentioned above and those derived from mutant G. For *FOB1* transformants, an efficient expansion of rDNA, that is, a large increase in the size of chromosome XII, was clearly observed for all the transformants derived from mutant A and G (Fig. 4B, lanes A and G). Some of them (one transformant of A and two transformants of G), however, showed lower degrees of expansion compared to others with the same mutation (A or G) or those without mutation (mentioned above). For mutant B, the extent of expansion was reduced significantly. However, two of the five *FOB1* transformants (marked with asterisks in Fig. 4B) showed a clear expansion and two others showed a smear, suggesting that at least some fractions of heterogeneous cell populations had started repeat expansion (Fig. 4B, lanes B).

For the other mutants, C, D, E, and F, no significant *FOB1*-dependent repeat expansion was observed. Only a limited increase in size was observed (Fig. 4A and B, lanes C, D, E, and F), and the patterns of chromosome XII bands shown by five *FOB1* transformants for each of these mutants were similar to those seen for vector transformants of the control strain, TAK201, or vector transformants of mutant G; that is, the bands were relatively homogeneous and could be recognized above the 1.1-Mb bands of chromosome VII and XV in the EtBr-stained gel (Fig. 4A). The absence of *FOB1*-dependent expansion was expected for mutant F because the RFB region has been completely replaced by the *URA3* gene in this mutant, and the model shown in Fig. 2 predicted this result. The results obtained for mutants C, D, and E demonstrate that there are DNA elements in these regions which are required for efficient *FOB1*-dependent expansion of rDNA repeats.

The *URA3* gene fragment which has replaced segments A to G individually in the NTS1 mutants A to G was found to undergo repeat expansion processes together with adjacent rDNA. In the experiment in Fig. 4, we rehybridized the same filter (A to G) with a *URA3*-specific probe after stripping the rDNA probe and obtained patterns of chromosome XII sizes similar to those shown in Fig. 4B (data not shown except for mutant G as an example in Fig. 4C). Hybridization with the *URA3* probe revealed bands of chromosome V, which carries a single copy of the native *URA3* gene (asterisk in Fig. 4C). Comparison of the intensities of chromosome XII bands with those revealed by the single-copy *URA3* show strong coamplification of *URA3* in *FOB1* transformants of mutant G and limited coamplification in vector transformants of mutant G.

In the CHEF electrophoresis experiments described above, it is difficult to obtain accurate estimates of the degree of rDNA repeat expansion. First, the conditions of electrophoresis were chosen to improve the resolution of chromosomal bands with different sizes at a region near 1.1 Mb, which made resolution of bands of 1.5 Mb or higher difficult (compare lane M in Fig. 4C with lane M in Fig. 3A). Second, significant fractions of chromosome XII failed to enter the gel, presumably reflecting the difficulty of obtaining complete release of this large chromosome from cellular components and/or debris resistant to enzyme digestion during sample preparation. Therefore, we determined the extent of increase of rDNA repeat numbers by Southern hybridization after digestion of DNA with *Bgl*II. Specific probes used to detect rDNA were probe 2 for mutant strains A to C and probe 1 for mutant strains D to G (indicated in Fig. 5C). A single-copy gene, *MCM2*, was also analyzed as a reference by using a suitable hybridization probe. The results obtained are shown in Fig. 5A for a single *FOB1* transformant taken from each group of mutants as well as single *FOB1* and single vector transformants of the control strain that were subjected to the rDNA repeat expansion process. First, it should be noted that the *Bgl*II fragment detected for the transformants derived from the control strain had a size of 4.6 kb (Fig. 5A, arrowhead marked rDNA), corresponding to the *Bgl*II-A fragment shown in Fig. 1A. The bands detected for the mutants were larger [Fig. 5A, arrowhead marked rDNA (URA3)], and no heterogeneity was observed for each mutant band. The larger sizes reflect the differences between the sizes of each region deleted (120 to 250 bp) and the size of the *URA3* fragment inserted (1.1 kb). The results demonstrate that each repeating unit in rDNA after extensive expansion (mutants A, B, and G [sample B shown in Fig. 5A was the one with a large expansion]) or after limited expansion (mutants C, D, E, and F) contained *URA3*; that is, *URA3* was coamplified with the remaining rDNA in the expansion process.

The number of rDNA repeats in the samples was determined by first measuring the intensities of bands, calculating the ratios of the rDNA to *MCM2* signals for each sample, and then comparing these ratios to the ratio obtained for the reference two-copy rDNA strain. This Southern analysis was repeated with the remaining four *FOB1* transformants of the mutants (A to G) and the control strain, as well as four vector transformants of the control strain. Averages of the values for five independent transformants obtained in this way were then calculated, and the results are shown in Fig. 5B. It is evident that replacing regions C, D, E, and F with *URA3* abolished the efficient *FOB1*-dependent repeat expansion. Repeat numbers were less than 10 in all these cases and were not larger than the small increases observed for the vector transformants of the control strain. In contrast, replacement of the A, B, and G segments with *URA3* still allowed *FOB1*-dependent expansion, although the extent of expansion appeared to be less than that observed for the control strain. In summary, the experiments described in this section demonstrate that the DNA region covering segments C to F is required for *FOB1*-dependent rDNA repeat expansion. We call this region EXP (for "expansion of rDNA repeats") (Fig. 5C).

Effects of NTS1 substitution mutations on replication fork-blocking activity. Although deletion analysis was previously carried out to define the region (the RFB region) required for RFB activity, the analysis was done by using artificial plasmid systems (3, 19) rather than in the context of the native rDNA locus on chromosome XII. To examine the relationship between the DNA elements required for rDNA repeat expansion and those required for RFB activity, we used the 2D electrophoresis method (1) and analyzed cultures of the five *FOB1*

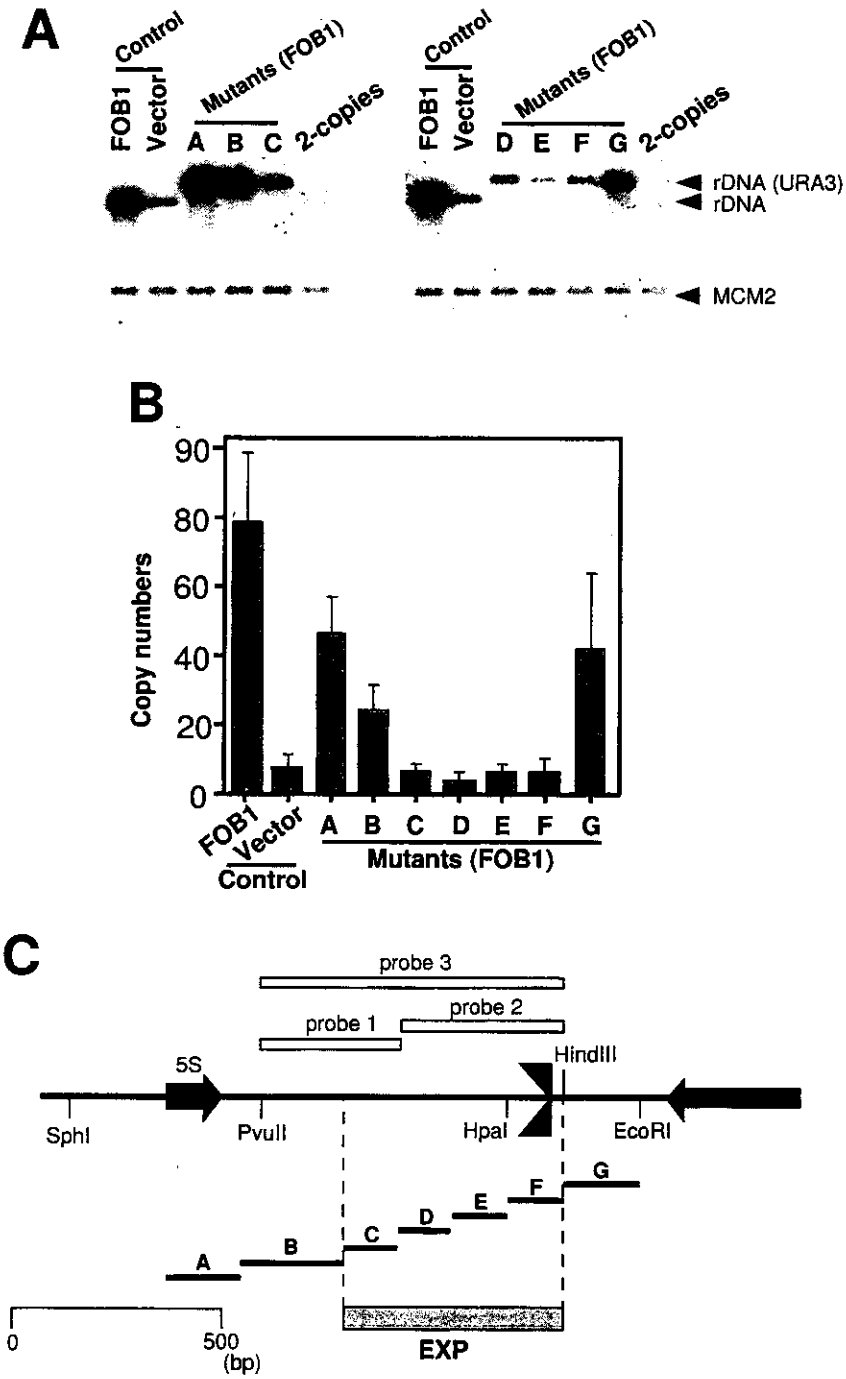

FIG. 5. Expansion of rDNA repeats observed in *FOB1* transformants of NTS1 substitution mutants. (A) The DNA samples analyzed in the experiments in Fig. 4A and B were digested with *Bgl*II and analyzed by Southern hybridization using rDNA-specific probes, probe 2 for the left panel and probe 1 for the right panel (the probes are indicated in panel C). The gels were also analyzed using a probe specific for a single-copy gene, *MCM2*, as a reference. (B) The numbers of rDNA repeats was calculated for each transformant, and the values for five independent transformants derived from each mutant and control strain were averaged. The results are shown as bars, and standard deviations are indicated as lines. (C) Summary of the mutational analysis indicating the region (EXP) essential for *FOB1*-dependent rDNA repeat expansion. The locations of segments A to G as well as probes 1, 2, and 3 used for hybridization are shown together with pertinent restriction sites in this region.

transformants of the mutants and the *FOB1* and vector transformants of the control strain (those used in the experiment in Fig. 5A) for accumulation of intermediates of replication arrested at the RFB site. DNA was isolated from cells growing exponentially in galactose medium, digested with *Bgl*II, and subjected to 2D gel electrophoresis followed by hybridization using a rDNA probe (probe 3 in Fig. 5C). The results are shown in Fig. 6. The control *FOB1* transformant culture

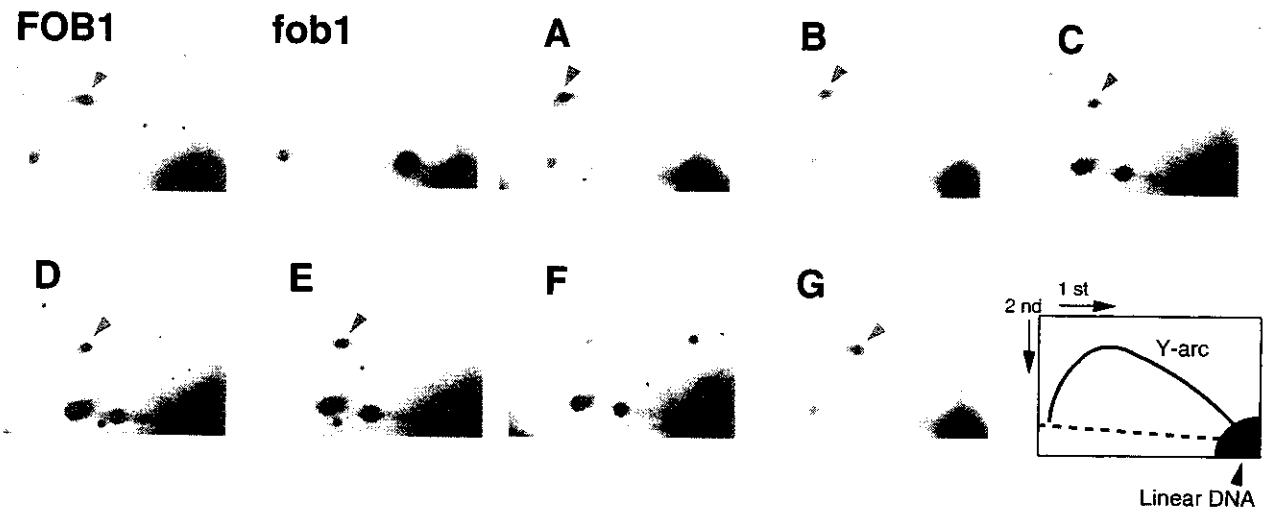

FIG. 6. Effects of NTS1 mutations on RFB activity analyzed by 2D gel electrophoresis. DNA was prepared from *FOB1* transformants of NTS1 substitution mutants (A to G) and *FOB1* and vector transformants (panels FOB1 and fob1, respectively) derived from the control strain, TAK201. DNA was then digested with *Bgl*II and subjected to 2D agarose gel electrophoresis followed by Southern hybridization using a rDNA probe (probe 3 in Fig. 5C). Spots indicated by arrowheads show the accumulation of Y-shaped molecules at RFB sites. A schematic diagram of the positions of various Y-shaped replication intermediates is shown as a Y-arc in the bottom right panel.

showed a spot (indicated by an arrowhead) corresponding to the replication fork intermediate arrested at the RFB site (panel FOB1). The vector transformant culture did not show such a spot (panel fob1), as expected from the previous work (20). For mutants A through E, a spot was observed at a position which is shifted slightly to the left from the position of the spot seen for the control *FOB1* cells (see the position of spots indicated by arrowheads in panels A through E relative to the position in panel FOB1). This small shift to the left is consistent with the increase in the size of the *Bgl*II A fragment caused by the *URA3* substitution (as mentioned above in connection with the results in Fig. 5A) combined with the expectation that the increase is in the replicated "branch" region of the Y-shaped intermediate formed at the site.

For mutant F, a large reduction in the spot intensity was observed, as expected from deletion of the previously defined RFB site in the F segment. However, we noted the presence of a weak spot at approximately the same position as those seen for mutants A to E, that is, slightly left of that seen for the control culture (compare panel F with other panels). Therefore, it appears that a weak RFB activity remains in mutant F and that the (weak) replication fork arrest takes place soon after replication of the *URA3* fragment inserted to replace the F segment, i.e., presumably in the G segment. Although further studies are required to establish this tentative conclusion, it is possible that we failed to detect this weak activity previously because the previous work was done with an artificial plasmid system, where the RFB activity was weaker than that observed in the chromosomal rDNA repeats (19).

When mutant G was analyzed, a clear spot was observed and its position was shifted to the right along the Y arc from the position observed for the control *FOB1* cells (panel G). This shift is consistent with replication fork arrest occurring at the previously defined RFB site in the F segment, that is, an increase in the size of the unreplicated "stem" of the Y-shaped replication intermediate in mutant G relative to the intermediate in the control *FOB1* cells.

The main conclusion obtained from the 2D gel analysis described above is that replacement of the C, D, or E segment with *URA3* does not affect the RFB activity even though it abolishes the *FOB1*-dependent expansion of rDNA repeats, as described in the previous section. Some specific DNA element(s) exists in the region covering segments C, D, and E (and perhaps extending to F) which is involved in a function(s) separate from replication fork blocking, enabling the expansion of rDNA repeats.

DISCUSSION

Identification of a new DNA *cis* element(s) required for expansion of rDNA repeats. We have constructed a yeast strain which carries only a single intact NTS1 region surrounded by two 5S-NTS2-35S regions on chromosome XII. The strain also carries a deletion in the *FOB1* gene, and an efficient *FOB1*-dependent repeat expansion can be induced by introduction of the missing *FOB1* gene. Using this system, we carried out a mutational analysis of the entire NTS1 region. We first confirmed the prediction based on the previously proposed model (Fig. 2) that the 129-bp *Hin*dIII-*Hpa*I region (segment F) containing the RFB site is required for rDNA repeat expansion. Although this confirmation does not necessarily prove the model (see the discussion below), the results are at least consistent with the proposal that replication fork blocking is required for the efficient expansion and contraction of rDNA repeats (18).

Somewhat unexpectedly, we have discovered that the adjacent ~400-bp region distal to the 35S rRNA gene (segments C, D, and E) is required for the *FOB1*-dependent repeat expansion even though it is not required for the RFB activity. Thus, this new *cis* element(s) defines a new function required for

expansion of rDNA repeats. Since both expansion and contraction are largely *FOB1* dependent (18), we think it likely that this new *cis* element, called EXP, is involved in both expansion and contraction, although the present experiments demonstrate only the requirement for expansion and not that for contraction. It should be noted that we define the EXP element (or region) as the DNA region required for repeat expansion, and this includes segment F, which contains the RFB region; regardless of whether replication fork blocking is really essential for repeat expansion, the RFB region is required for expansion and hence is included in the EXP region. If replication fork blocking is really essential for efficient *FOB1*-dependent repeat expansion, the EXP region would be functionally divided into two subregions or DNA elements, one required for replication fork blocking and the other required for another function, a function presumably involved in a step subsequent to replication fork blocking, and these two elements might or might not overlap in segment F.

Regarding the function of the EXP element that is independent of the RFB function, we have little information. As discussed previously (18, 25), there are two different kinds of factors which influence rDNA repeat expansion and contraction. One comprises protein factors which are involved in recombination processes, such as Fob1 protein and Sir2 protein (in addition to proteins used in recombination in general, such as *RAD52* [T. Kobayashi, unpublished data]), and the other includes protein factors, such as Pol I and Pol I-specific transcription factors, which presumably do not participate in recombination processes but do participate in the maintenance of rDNA repeat numbers within a certain range, presumably by forming some specific nucleolar structures that include rDNA repeats. For example, in mutants defective in Pol I and growing by Pol II-dependent transcription of an artificial fusion gene, *GAL7*-35S rDNA, on a plasmid, rDNA repeat numbers are reduced to about half of the normal level (18). Another example is that of mutants defective in the transcription factor UAF and growing by transcribing chromosomal rDNA by Pol II, where average rDNA repeat numbers are increased to approximately 400 (25, 36). In both instances, average repeat numbers are substantially altered relative to the wild-type level but the cells retain the ability to expand and contract rDNA repeats and the populations show a significant heterogeneity of cells with different sizes of rDNA repeats. In mutants C, D, and E, which fail to expand rDNA repeats, a limited degree of expansion was observed but the repeat numbers appeared to be relatively homogeneous. Repeat numbers obtained were also different among five different transformants for a given mutation. Therefore, it appears that the EXP element defined here is involved in a *FOB1*-dependent recombination process(es) rather than influencing repeat numbers through some nucleolar structures. Since the replacements of each of segments C, D, and E (but not A, B, and G [see below]) with the *URA3* sequence all abolish *FOB1*-dependent repeat expansion, we suspect that the EXP element may represents a site(s) for the binding of some specific protein factor(s) that is involved in a recombination process(es) unique to rDNA repeats, possibly the one(s) counteracting the function of other rDNA-specific chromatin proteins, such as Sir2 protein, that decrease the frequency of recombination within rDNA repeats.

Replacement of each of segments A, B, and G with *URA3* allowed *FOB1*-dependent rDNA repeat expansion, but the extents of repeat number increase after 45 generations were significantly lower than for the control. Since the degrees of expansion were not uniform in five *FOB1* transformants analyzed for each of these mutants, these mutations appear to reduce the rate of expansion rather than limiting the extent of expansion. It is possible that some DNA sequence elements contained in these segments play some specific (stimulatory) role in repeat expansion but are not essential. Alternatively, the presence of a Pol II gene, *URA3*, in each repeat that would attract nonnucleolar proteins including the Pol II transcription machinery may cause a nonspecific inhibition of the expansion process.

In a search of genomic DNA elements that would promote the amplification of a plasmid carrying the thymidine kinase gene in cultured mouse cells, Wegner et al. (38) isolated two DNA fragments (called muNTS1 and muNTS2) which were identified as two segments within the nontranscribed spacer region in rDNA repeats. The plasmids carrying these sequences were found to be integrated into some chromosome locations in the form of long tandem repeats. Because these two nontranscribed spacer fragments were highly AT rich, a likely possibility considered was that they might function as origins of replication. The fragments in the EXP region studied here contain some AT-rich sequences, but other fragments that were not required for expansion (A, B, and G) also contain equally AT-rich segments. In addition, the function of the EXP element in the yeast system is clearly not that of a replication origin. Whether there is any functional relationship between the mouse nontranscribed spacer elements and the yeast EXP element is presently unclear.

***FOB1*-independent limited increases of rDNA repeats.** The system we used to study *cis* elements for rDNA repeat expansion contains a single NTS1 surrounded by two copies of 5S-NTS2-35S repeats. The *FOB1*-dependent expansion model in Fig. 2 requires at least three tandem repeats for repeat expansion. Therefore, expansion in the present system must have initially used a different mechanism, such as an unequal crossing over between sister chromatids. A limited degree of *FOB1*-independent expansion was in fact observed in vector transformants of the control strain (and of NTS1 mutants). The RFB-independent limited expansion observed in *FOB1* transformants of mutant F may also represent such a *FOB1*-independent repeat expansion. Although we have not studied mechanisms involved in such *FOB1*- and RFB-independent copy number increase, this process is presumably very inefficient because of the small numbers of repeats available as sites of recombination and the general reduction of recombination caused by protein components unique to rDNA chromatin, such as the Sir2 and Net1 proteins (10, 32). We expect that once copy numbers reach certain sizes, *FOB1*-dependent repeat number alterations will start to become dominant and the rate of expansion per genome may presumably become higher with increased copy numbers during the 45 generations used for the analysis, because an increase in repeat number will increase the total frequency of *FOB1*-dependent recombinational events. In addition, the direction of copy number changes in populations will be mostly toward expansion rather than contraction due to a selection for faster growth, at least until certain repeat numbers are reached (see below). Such