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Saccharomyces cerevisiae Srs2 DNA Helicase Selectively Blocks Expansions of Trinucleotide Repeats

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Trinucleotide repeats (TNRs) undergo frequent mutations in families afflicted with certain neurodegenerative disorders and in model organisms. TNR instability is modulated both by the repeat tract itself and by cellular proteins. Here we identified the *Saccharomyces cerevisiae* DNA helicase Srs2 as a potent and selective inhibitor of expansions. *srs2* mutants had up to 40-fold increased expansion rates of CTG, CAG, and CGG repeats. The expansion phenotype was specific, as mutation rates at dinucleotide repeats, at unique sequences, or for TNR contractions in *srs2* mutants were not altered. Srs2 is known to suppress inappropriate genetic recombination; however, the TNR expansion phenotype of *srs2* mutants was largely independent of *RAD51* and *RAD52*. Instead, Srs2 mainly functioned with DNA polymerase delta to block expansions. The helicase activity of Srs2 was important, because a point mutant lacking ATPase function was defective in blocking expansions. Purified Srs2 was substantially better than bacterial UvrD helicase at in vitro unwinding of a DNA substrate that mimicked a TNR hairpin. Disruption of the related helicase gene *SGS1* did not lead to excess expansions, nor did wild-type *SGS1* suppress the expansion phenotype of an *srs2* strain. We conclude that Srs2 selectively blocks triplet repeat expansions through its helicase activity and primarily in conjunction with polymerase delta.

Trinucleotide repeats (TNRs) undergo frequent expansions in families afflicted with Huntington's disease, fragile X syndrome, and at least 13 other neurodegenerative diseases (7, 8, 35). In addition to this biomedical relevance, the unusual genetic mechanisms underlying TNR instability have sparked substantial scientific interest. Studies in the field of human genetics and with model organisms indicate that the TNR DNA itself plays a major role in its own mutability (7, 8, 17, 35, 45). For example, the risk of expansion is closely tied to *cis*-acting features, such as the sequence and length of the TNR tract, and whether the repeat is perfect or imperfect. While these "rules" can usually predict expansion risk, there are clear exceptions. For example, the risk of an expansion can vary greatly depending on whether the gene is inherited maternally or paternally (35, 45). In addition, genetic background and other factors have been suggested to explain, for example, the variations in germ line instability among individuals with similar allele lengths at the Huntington's disease locus (22).

These observations provide some of the evidence suggesting that cellular proteins modulate the likelihood of TNR instability. What proteins might be involved? Important clues come from models of expansion (7, 12, 17, 45). While expansions result from more than one genetic mechanism, a central feature of essentially all expansion models is that single-stranded TNR sequences fold into aberrant DNA structures, usually hairpins (11), which are crucial intermediates in the mutation process (12, 17, 45). If a hairpin cannot be prevented from forming or is not removed quickly enough, an expansion will ensue. Thus, hairpins are thought to be direct precursors of expansions. Proteins that either prevent hairpin formation or accelerate hairpin removal would help reduce expansion rates. One well-characterized protein that helps block hairpin formation is the flap endonuclease FEN-1 (24), which removes some single-stranded TNR flaps (10, 24, 25, 40, 43, 44). However, it is likely that other proteins also help avoid expansions.

In this study, the Srs2 DNA helicase was identified in a genetic screen for inhibitors of expansion in *Saccharomyces cerevisiae*. Characterization of *srs2* mutants indicates that the helicase is a potent and selective inhibitor of TNR expansions.

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MATERIALS AND METHODS

Strains. Most of the *S. cerevisiae* strains used were derived from MW3317-21A (18) (*MAT*α Δ*trp1 ura3-52 ade2*Δ *ade8 hom3-10 his3-Kpn1 met4 met13*). Isogenic derivatives containing disruptions of *msh2*, *rad27*, *rad52*, or *rad51* were constructed by standard techniques (26, 39) and confirmed by Southern blotting and phenotypic analysis. The strain harboring the *srs2*::*TRP1* mutation, HKY723-5D (*MAT***a** *hpr5*::*TRP1 leu2-3,112 his3-11*, *15 ade2-2 ura3-1 trp1-1 can1-100 RAD5*$^+$), was kindly provided by Hannah Klein, New York University School of Medicine. A strain harboring *sgs1*::*kanMX4* was obtained from Open Biosystems. BL 492, a *pol32*Δ derivative of BL490 (*MAT***a** *leu2*Δ*1 trp*Δ*63 ura3-52 his3-200*) was a generous gift from Sergei Mirkin (University of Illinois, Chicago). The chromosomal integration of TNR-containing plasmids and confirmation of correct single integrants were done as described previously (33, 37).

Plasmids. All triplet repeat-containing plasmids were constructed with the pBL94 vector as described previously (38). Plasmids pHK209 (BamHI-SalI insert of *SRS2* in YCp50), pHK282 (EcoRI-SalI insert of *srs2K41A* in pRS314) and pFP56 (*srs2*::*TRP1*) were generous gifts from Hannah Klein. To create low-copy-number p*SRS2* plasmids, the *SRS2* coding fragment was recovered as a BamHI-SalI fragment from pHK209 and subcloned into pRS314 (marked with *TRP1*) or pRS315 (marked with *LEU2*) (42). When low-copy-number p*SRS2* was used for complementation studies of the *srs2*::*LEU2* and *srs2*::*TRP1* alleles, the appropriate p*SRS2* plasmid was always added to the strain already containing the triplet repeat reporter. The high-copy-number p*SRS2* plasmid used for overexpression studies was created by transfer of the *SRS2* gene as an EcoRI-SalI fragment from pHK209 into pRS424 (6). The reporter plasmid pSH44, containing 16.5 repeats

of the dinucleotide repeat GT (14), was kindly provided by Tom Petes, University of North Carolina. The 2μm plasmid YEplac195-*SGS1* was a generous gift from Alan Morgan, University of Liverpool. For this study, the *SGS1* gene was moved as a SalI fragment into the corresponding site of the high-copy-number plasmid pRS424.

Genetic assays and molecular analysis of mutated TNR alleles. Expansion and contraction rates were measured by fluctuation analysis as described previously (33, 37). Briefly, TNR tracts were cloned into a yeast promoter-reporter to create spacing-sensitive expression of a downstream *URA3* gene. Tracts containing up to 25 TNRs or a subset of repeats plus randomized sequence DNA equivalent to 25 repeats were used to score expansions of +5 repeats or more (33, 37). To measure contraction rates, the assay is performed with longer starting tracts (here we used 25 repeats plus 24 random-order nucleotides, equivalent to eight repeats), referred to as CTG_{25+8} (37) Contractions of −5 or more repeats are identified as Ura^+ colonies. Dinucleotide repeat mutation rates were measured as described previously (14). Forward mutation rates for the *CAN1* gene were determined by selection for canavanine resistance. Three to five independent clones were tested for each of the above assays to ensure reproducibility. Single-colony PCR analysis of TNR expansions and contractions was performed by a published method (33). The percent bona fide expansions or contractions, determined by PCR analysis, was multiplied by the apparent expansion and contraction rates derived from fluctuation analysis. All rates reported here reflect this correction factor.

Western blot analysis. The expression of Srs2 was analyzed by Western blotting. Briefly, 10^7 cells/ml from a 5-ml overnight culture were harvested and lysed in sample buffer with glass beads; 10 μl of this lysate (containing 5 μg of total protein) was loaded onto a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 7.5% polyacrylamide gel and separated by electrophoresis, the proteins were transferred to a nitrocellulose membrane (Amersham), and the membrane was blocked overnight at 4°C in Odyssey blocking buffer (Li-Cor). Immunodetection was done with rabbit anti-Srs2 antiserum (from Patrick Sung, Yale University) or with anti-Rfc3 antiserum (from Peter Burgers, Washington University), followed by probing with Alexa Fluor 680 goat anti-rabbit immunoglobulin G (Molecular Probes) and fluorescence detection and quantitation with the Odyssey system, per the manufacturer's protocol.

Helicase substrate analysis and unwinding assays. End labeling with ^{32}P by T4 polynucleotide kinase (New England Biolabs) was performed according to the manufacturer's protocol. Oligonucleotide strands (one radiolabeled) were mixed at equimolar concentrations with the unlabeled complement, heated at 95°C for 5 min, and then slowly cooled to room temperature. The location of the radiolabel on either the longer or shorter strand is stipulated in the legends to Fig. 3 and 4. Mung bean nuclease (New England Biolabs) digestions (total volume, 20 μl) were performed with 1 unit of enzyme in the manufacturer's reaction buffer at 37°C for 40 min. Reactions were quenched by addition of SDS to 0.01% final concentration, followed by ethanol precipitation, resuspension in formamide loading buffer, and analysis on a 15% sequencing gel.

For helicase assays, the radiolabeled partial duplex DNA substrate was incubated with 25 to 35 nM purified Srs2 for 0 to 15 min under standard helicase assay conditions; 30°C in the presence of 300 nM (nucleotides) DNA substrate, 25 mM Tris-Cl (pH 7.5), 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg of bovine serum albumin per ml, and 2 mM ATP (19). UvrD was used at 200 nM for 0 to 20 min as follows (30); the reaction mixture contained 25 mM Tris-Cl (pH 7.5), 3 mM $MgCl_2$, 20 mM NaCl, 50 mM 2-mercaptoethanol, 50 μg of bovine serum albumin per ml, and 10 nM DNA substrate. UvrD was diluted in helicase II storage buffer and then incubated for 5 min at 37°C with the reaction mix lacking ATP. The unwinding reaction was initiated by addition of ATP to a final concentration of 3 mM. For all helicase reactions, the DNA products were subsequently analyzed by nondenaturing 15% PAGE.

RESULTS

Identification of *SRS2* as a gene affecting triplet repeat expansions. To find candidate genes that help protect the cell from expansions, random transposon mutants of *S. cerevisiae* were screened for increased expansions of a $(CTG)_{13}$ reporter (4). A CTG sequence was used because CTG•CAG tracts are unstable in several human genetic diseases. The length of 13 is relevant in *S. cerevisiae* because it falls close to a crucial threshold length (37) at which expansions become increasingly more frequent. We reasoned that yeast mutations that alter $(CTG)_{13}$ expansion rates might be specific for TNRs, since thresholds are unique to TNRs. Thresholds are well known in human TNR instability, where lengths of ≈35 repeats are associated with increased risk of expansion (35).

DNA sequencing (4) identified *srs2*::*LEU2* as a disrupted gene that gave increased expansion rates. Additional genetic assays were performed to verify the *srs2* mutation. Both the *srs2*::*LEU2* mutant and an *srs2*::*TRP1* mutant showed moderate UV sensitivity, consistent with published results (1). Nonhomologous end joining, measured by a plasmid repair assay, was reduced 2.5-fold in the *srs2*::*LEU2* background, in good agreement with published values (13). Identical expansion phenotypes were observed with both the *srs2*::*LEU2* and the *srs2*::*TRP1* strains, and a low-copy-number number p*SRS2* plasmid reversed the hyperexpansion phenotype of the *srs2*::*LEU2* mutation (see below). Together with the sequencing data, these results verified *SRS2* as the mutated gene.

The hyperexpansion phenotype of the *srs2* mutant was surprising because neither spontaneous nor damage-induced mutagenesis, measured as forward mutations at *CAN1*, is increased in *srs2* strains (2). To evaluate the hyperexpansion phenotype more thoroughly, quantitative mutation rates were determined. Expansions of the $(CTG)_{13}$ tract (37) were elevated 40- to 44-fold over that in the wild type for our *srs2*::*LEU2* allele and for the *srs2*::*TRP1* strain obtained from another laboratory (Table 1). The wild-type *SRS2* gene on a low-copy-number plasmid (p*SRS2*) reduced the expansion rate to wild-type levels. The expansion sizes of CTG tracts in *srs2*::*LEU2* were also measured (33). Starting from an initial $(CTG)_{13}$ tract, the expanded alleles ranged from +5 to +10 repeats, with a median of +8, similar to expansions in the wild-type strain (Fig. 1). As the two mutational spectra overlap, we conclude that the number of expansions, not their size, accounts for the dramatic increase in the expansion rate of the *srs2* strain. There was a single case of a large expansion (+31 repeats) in the *srs2*::*LEU2* mutant (Fig. 1). However 98% of CTG expansions in the *srs2* strain were less than twice the original tract size, consistent with a replicational model of TNR repeat instability (12).

Hyperexpansion phenotype of an *srs2* mutant is mainly associated with DNA polymerase delta, not with genetic recombination. Genetic and biochemical studies (1, 3, 19, 47) indicate that Srs2 functions as an antirecombinase, and many *srs2* phenotypes are linked to recombination (16). Therefore, it was important to know whether the hyperexpansion phenotype of *srs2* was also a result of hyperrecombination. If so, expansion rates should be reduced to near wild-type levels when recombination is inactivated. In contrast to this prediction, the results in Table 1 show that double mutants (*rad51 srs2* and *rad52 srs2*) still retained much of the instability (11- to 33-fold) seen in an *srs2* single mutant (40- to 44-fold). Thus, loss of recombination reduced the *srs2* effect on CTG expansions by only 1.3- to 4-fold. While a portion of the *srs2* expansion phenotype might be due to recombinational misprocessing of TNRs, some other process is responsible for the majority of the phenotype. Expansion rates were also unaffected when either *rad51* or *rad52* was mutated singly (Table 1), arguing against unequal sister chromatid exchange (9) as a mechanism of expansion.

An alternative possibility is that Srs2 functions with DNA polymerase delta to block expansions. This idea is based on the

TABLE 1. Mutation rates in *srs2* mutant strains[a]

Mutation (exponent) and genotype	Mean no. of mutations/ cell generation, 10^{-n} (± SD)	Ratio (fold over wild type)
Expansions of $(CTG)_{13}$ (10^{-7})		
SRS2	1.4 (± 0.2)	1.0
srs2::*LEU2*	56 (± 11)	40
srs2::*TRP1*	62 (± 11)	44
srs2::*LEU2* + *pSRS2*	1.8 (± 0.8)	1.3
rad51::*kanMX*	1.3 (± 0.5)	0.9
srs2::*TRP1 rad51*::*kanMX*	16 (± 0.6)	11
rad52::*LEU2*	≈1	≈0.7
srs2::*TRP1 rad52*::*LEU2*	46 (± 5)	33
*pol32*Δ	41 (± 3)	29
srs2::*LEU2 pol32*Δ	28 (± 12)	20
*pol32*Δ + high-copy *SRS2*	2.0 (± 0.5)	1.4
srs2::*LEU2* + *psrs2K41A*	24 (± 9)	17
sgs1::*kanMX4*	2.4 (± 0.5)	1.7
srs2::*LEU2* + high-copy *SGS1*	60 (± 11)	43
Expansions of $(CTG)_{25}$ (10^{-5})		
SRS2	1.0 (± 0.3)	1.0
srs2::*LEU2*	4.9 (± 0.8)	4.9
srs2::*TRP1*	5.1 (± 1.1)	5.1
srs2::*LEU2* + low-copy *pSRS2*	1.4 (± 0.2)	1.4
Expansions of $(CAG)_{25}$ (10^{-7})		
SRS2	<5	1.0
srs2::*LEU2*	23 (± 0.7)	4.6
Expansions of $(CGG)_{25}$ (10^{-5})		
SRS2	6.8 (± 0.7)	1.0
srs2::*LEU2*	23 (± 5)	3.4
Dinucleotide repeat mutations (10^{-5})		
SRS2	3.8 (± 0.2)	1.0
srs2::*LEU2*	4.3 (± 0.3)	1.1
*msh2*Δ	1,400 (± 10)	370
CAN1 forward mutations (10^{-7})		
SRS2	3.0 (± 0.4)	1.0
srs2::*LEU2*	3.7 (± 0.3)	1.2
*rad27*Δ	180 (± 10)	60
Contractions of $(CTG)_{25+8}$ (10^{-5})		
SRS2	2.7 (± 0.2)	1.0
srs2::*LEU2*	2.5 (± 0.3)	0.9
srs2::*TRP1*	2.2 (± 0.4)	0.8

[a] Rates were determined by the method of the median (20). Rates are the averages of three to six determinations for genetically independent clones. The exponent is shown separately for each section. The ratio value is the mutation rate for the indicated genotype divided by the wild-type rate.

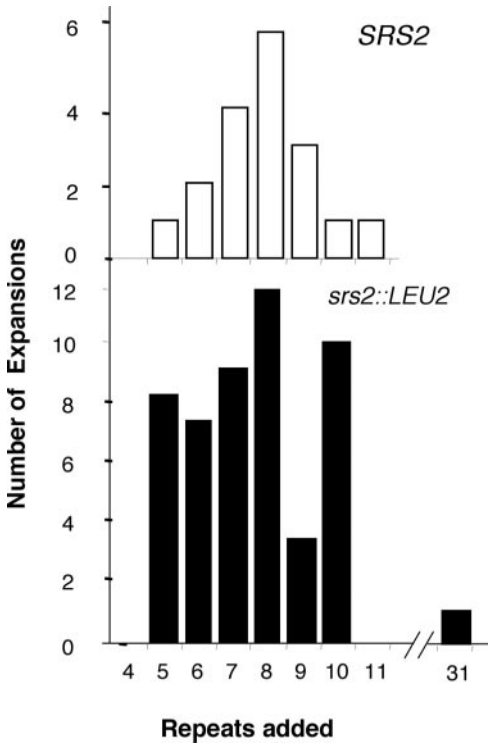

FIG. 1. $(CTG)_{13}$ expansion sizes in wild-type and *srs2*::*LEU2* strains. A subset of the 5-fluoroorotic acid-resistant colonies obtained from fluctuation analysis were subjected to single-colony PCR analysis and sizing on sequencing gels to an accuracy of ±1 to 2 repeats (33). The *x* axis denotes the number of repeats added to the original tract of 13 repeats.

finding that Srs2 interacts in two-hybrid experiments with the Pol32 subunit of polymerase delta (15). If Srs2 and polymerase delta are both needed for inhibiting TNR expansions, then a mutant lacking *POL32* should show a high expansion rate. A $(CTG)_{13}$ tract was 29-fold more unstable in a *pol32*Δ strain than its wild-type counterpart (Table 1). Similarly, the repeat tract was 20-fold destabilized in an *srs2 pol32* double mutant. This epistasis analysis is consistent with the idea that the majority of expansions prevented by Srs2 occur in conjunction with Pol32, and hence polymerase delta. Statistical analysis by Student's *t* test showed no significant differences between the expansion rates for *srs2* compared to *pol32*Δ ($P = 0.08$) or for *pol32*Δ compared to *srs2 pol32*Δ ($P = 0.14$); however, there was a significant difference between *srs2* and *srs2 pol32*Δ ($P = 0.04$).

The epistasis relationship between Srs2 and polymerase delta may be somewhat complex. To examine this idea further, we tested the effect of Srs2 overexpression. If a direct physical interaction between Srs2 and Pol32 is important for preventing expansions (for example, to recruit Srs2 to a replication fork), overexpressing Srs2 in a *pol32*Δ background should not alleviate the hyperexpansion phenotype. In contrast to the prediction, we found that the expansion rate for Srs2 overexpression in a *pol32*Δ strain was indistinguishable from wild-type levels (Table 1). Immunoblotting (Fig. 2B) showed that Srs2 levels were elevated three- to fourfold when cells harbored *SRS2* on a multicopy plasmid. We conclude that Srs2, when overexpressed, does not require physical interaction with Pol32 to block expansions. Caution is warranted when interpreting this result, however, because the interaction may be more important when Srs2 is present at normal levels.

Srs2 selectively blocks TNR expansions. Expansion rates were also examined for a longer TNR, $(CTG)_{25}$. In a previous study (37), we showed that $(CTG)_{25}$ is above the apparent threshold length of 13 repeats in *S. cerevisiae*. As a reference, wild-type cells undergo expansions of $(CTG)_{25}$ 70-fold more often than for $(CTG)_{13}$ (37). It was therefore of interest to know whether Srs2 can block expansions of a TNR above the threshold, where expansions occur at a relatively high rate even in the wild type. We found that the absence of Srs2 further increased instability for $(CTG)_{25}$. Expansion rates (Table 1) were approximately fivefold higher in the *srs2*::*LEU2* and *srs2*::*TRP1* mutants compared to the wild type, and the low-copy-number p*SRS2* plasmid complemented the *srs2*::*LEU2* mutation. Thus, Srs2 still provides protection against expansions for longer CTG tracts. Two other TNRs capable of forming hairpins, $(CAG)_{25}$ and $(CGG)_{25}$, showed 3.4- to 4.6-fold enhancement of expansion rates in an *srs2* mutant, respectively (Table 1), suggesting that Srs2 suppresses expansions for at

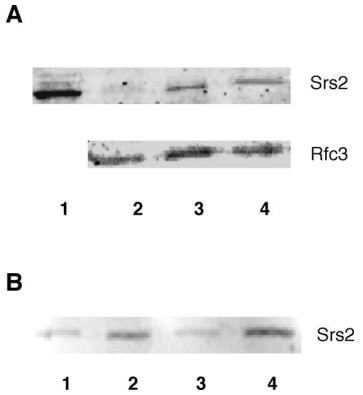

FIG. 2. Immunoblot analysis of Srs2 expression. Whole-cell extracts were separated by SDS–7.5% PAGE, transferred to a nitrocellulose filter, blotted with polyclonal Srs2 antiserum, and detected by fluorescence. (A) Lane 1, purified Srs2. Lane 2, extract from *srs2*::*LEU2* cells. Lane 3, extract from *srs2*::*LEU2* cells containing low-copy-number p*SRS2* plasmid. Lane 4, extract from *srs2*::*LEU2* cells containing the low-copy-number mutant plasmid p*srs2K41A*. Bottom strip, loading control immunoblot for Rfc3. Similar results were seen in two other repetitions of this experiment. (B) Lane 1, extract from wild-type cells. Lane 2, extract from wild-type cells containing a high-copy-number p*SRS2* plasmid. Lane 3, extract from *pol32*Δ cells. Lane 4, extract from *pol32*Δ cells with a high-copy-number p*SRS2* plasmid. A similar result was seen in a repetition of this experiment.

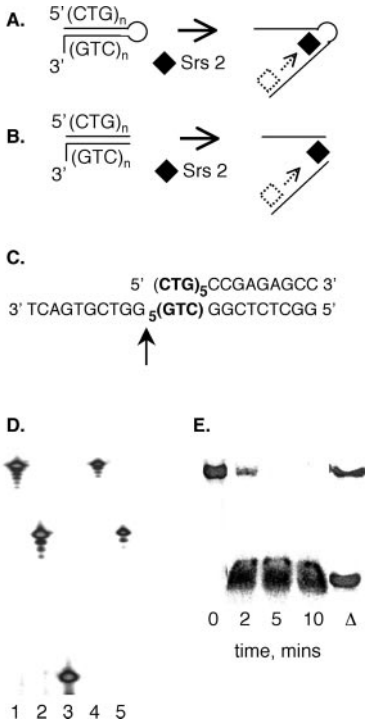

FIG. 3. Srs2 unwinds CTG repeat-containing double-stranded DNA. (A) Schematic diagram of putative Srs2 action on a CTG hairpin in vivo. In the diagram, a newly synthesized CTG tract has folded onto itself to form a hairpin (the template strand has been omitted for clarity). Srs2 (diamond) loads onto the 3′ DNA end and unwinds the structure. The single-stranded product might then reanneal properly to the template strand or might be subject to nuclease digestion, followed by resynthesis of the tract. Either outcome would help eliminate hairpins and thereby reduce the appearance of expansions. (B) Partial DNA duplexes containing CTG repeats can be tested in vitro as potential Srs2 helicase substrates. Srs2 action is envisioned to work as in A. The products of unwinding are single-stranded DNAs which can be readily assayed by gel electrophoresis. (C) Predicted structure of the partially double-stranded substrate. The 3′ tail of the longer strand provides a loading site for the helicase. The duplex contains five CTG repeats to mimic a TNR hairpin. The nine complementary base pairs add thermodynamic stability to the duplex and help ensure that the CTG repeats align as predicted. The arrow shows the predicted junction between single- and double-stranded DNA; 24 nucleotides of the bottom strand should be protected from mung bean nuclease, an enzyme that cleaves single-stranded DNA but not double-stranded regions. (D) Nuclease analysis of DNA structure. The annealed substrate, 5′-end labeled on the longer strand with ^{32}P, was incubated with mung bean nuclease. The products were analyzed on a denaturing 15% polyacrylamide gel. Lanes 1, 2, and 3 contain molecular size markers of 34, 24, and 9 nucleotides, respectively. Lane 4 contains the undigested helicase substrate, and lane 5 contains the product after nuclease digestion. (E) Srs2 helicase activity. The DNA substrate, 5′-end labeled on the shorter strand with ^{32}P, was incubated under standard helicase assay conditions (Materials and Methods) with 50 nM Srs2 for the indicated times. Δ, heat-denatured control.

least three different TNR sequences. In contrast, *srs2* had no effect on the non-hairpin-forming and genetically stable repeat $(CTA)_{25}$ (see supplemental material at http://www.unmc.edu/Eppley/publications/chart_lah3.html). Similarly, there was no detectable increase in mutation rates for poly(GT) dinucleotide repeats (Table 1) or for forward mutations that inactivate the *CAN1* gene (Table 1). Together, these results indicate a high degree of specificity for Srs2 on TNR sequences capable of hairpin formation.

TNRs also undergo contractions, in which repeating units are deleted. It is thought that contractions also occur via a hairpin-dependent mechanism, although a hairpin on the template strand is predicted to lead to contractions, not on the daughter strand, as for expansions. There was no significant effect of *srs2* on contractions of a $(CTG)_{25}$ reporter (37) (Table 1). Contractions of a shorter tract, $(CTG)_{15}$ (37), were also essentially unaffected by the *srs2* mutation ($3.9 \times 10^{-6} \pm 1.2 \times 10^{-6}$ versus $2.4 \times 10^{-6} \pm 0.6 \times 10^{-6}$ for the wild type). These results suggest that Srs2 acts primarily on the daughter DNA strand, not the template strand, to block triplet repeat instability. Alternatively, the mechanisms of expansion and contraction might be somewhat different and therefore differentially sensitive to the loss of Srs2, even though hairpins likely mediate both types of mutation.

Srs2 helicase activity is important for preventing expansions. Disease-causing TNRs form hairpins (11) that are likely precursors of expansions (12, 17, 45). Since Srs2 has helicase activity (2, 16, 19, 47), we hypothesized that Srs2 helps prevent expansions by unwinding hairpins. If so, the ATPase activity of Srs2 should be important for blocking expansions in vivo. We found that an *srs2* point mutant lacking ATPase function, *srs2K41A* (19), gave a $(CTG)_{13}$ expansion rate within about twofold of that of the null allele (Table 1). The expression levels of the wild-type and Srs2K41A proteins were similar (Fig. 2A), so the expansion defects in the K41A mutant cannot be attributed to low expression.

If Srs2 acts in vivo to unwind triplet repeat hairpins and prevent expansion, then perhaps the activity can be mimicked in vitro with double-stranded DNA containing CTG repeats within the duplex region (Fig. 3A and B). A DNA substrate was designed to test this idea (Fig. 3C), based on the following

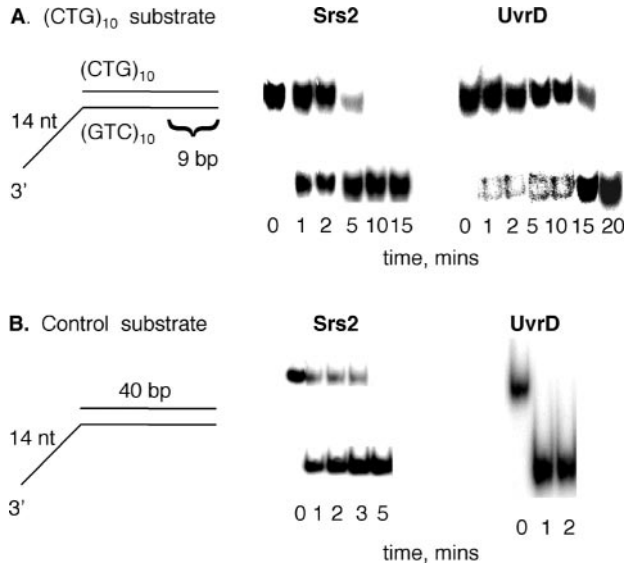

FIG. 4. Srs2 unwinds a TNR substrate substantially faster than does UvrD. DNA substrates radiolabeled on the shorter strand were incubated with either Srs2 (35 nM) or UvrD (200 nM) under standard conditions (19, 30) for the indicated times, and the reaction products were separated by PAGE and visualized by phosphorimaging. Both DNA substrates contained a 14-nucleotide-long 3′ tail, which is usable by either Srs2 or UvrD. (A) Schematic diagram of the $(CTG)_{10}$-containing substrate. Along with the 9 bp of nonrepeating DNA (identical to Fig. 3C), the duplex region is 39 bp in total length. (B) Control substrate (19) without triplet repeats. The duplex region measures 40 bp.

rationale. A single-stranded $(CTG)_{13}$ repeat tract (the presumed intermediate during expansion in vivo) can fold into a hairpin with maximally five to six repeats on one side of the stem, a short loop, and then five to six repeats on the other side of the stem. Thus, a helicase substrate with five CTG repeats (Fig. 3C) is reasonable. Control experiments indicated that the molecule adopted the predicted structure (Fig. 3D). Srs2 helicase readily unwound the DNA substrate (Fig. 3E) in an ATP-dependent manner (data not shown). The fact that Srs2 was able to unwind duplex DNA containing CTG repeats supports the idea that Srs2 prevents expansions by unwinding TNR hairpins.

To address the specificity of helicase action, we compared Srs2 to UvrD from *Escherichia coli*. UvrD protein, also called DNA helicase II, was chosen for comparison because it has been well studied (29, 31, 32) and has the same 3′-to-5′ polarity (28) as Srs2. If Srs2 is especially active at unwinding TNR-containing substrates, then perhaps there would be a difference in helicase activity compared to UvrD. Figure 4A demonstrates the activity of both enzymes on a $(CTG)_{10}$ partial duplex. Srs2 completely unwound the substrate by the 10-min time point, approximately twice the time required to unwind $(CTG)_5$ (compare Fig. 4A and 3E). In contrast, bacterial UvrD proceeded slowly through the $(CTG)_{10}$ substrate (Fig. 4A), requiring 20 min for full unwinding despite being assayed at a higher enzyme concentration (200 nM for UvrD versus 35 nM for Srs2). Both enzymes were active on a control substrate without repeats (Fig. 4B). The difference in UvrD's activity cannot be attributed to different duplex lengths, since the total duplex region in the two substrates was 39 and 40 bp long. The findings in Fig. 4 suggest that either the sequence or the structure or both of the $(CTG)_{10}$ substrate presents a barrier to unwinding that can be overcome by Srs2 faster than by UvrD, another 3′-to-5′ helicase.

We also asked whether a related yeast helicase, Sgs1, could substitute for Srs2 in vivo. Sgs1 is a member of the RecQ family of DNA helicases with 3′-5′ polarity (16). *SRS2* and *SGS1* mutants share several phenotypes, but single mutants also show differences (16). Although *sgs1* mutants show increased gross chromosomal rearrangements (34), an *sgs1*::*kanMX4* strain in our assay gave a low expansion rate that was very close to the wild-type value (Table 1). Thus, *sgs1* and *srs2* mutants have different phenotypes for TNRs. In some circumstances, *SGS1* in high copy number can suppress *srs2* phenotypes (27). However, a multicopy plasmid containing *SGS1* (27) failed to suppress the expansion defect conferred by *srs2*::*LEU2* (Table 1). Our results that *SRS2* and *SGS1* behave differently at TNRs are in general agreement with those of White et al. (48), who found that an *sgs1* mutation caused stabilization (not destabilization) of CGG arrays. These results suggest that Srs2 plays a unique role in stabilizing triplet repeat tracts that cannot be replaced by the functionally similar protein Sgs1.

DISCUSSION

We identified *srs2* in a blind screen for mutations that result in high expansion rates. Subsequent analysis showed that CTG, CAG, and CGG repeats are destabilized in *srs2* mutants. The *srs2* mutator phenotype is specific for TNR expansions, as there was no detectable increase in *srs2* backgrounds for TNR contractions, dinucleotide repeat alterations, or mutations in unique DNA sequences. Epistasis analysis indicated that the *srs2* expansion phenotype was mostly associated with DNA polymerase delta, with only a fraction of the expansion rate in *srs2* mutants attributable to recombination. Expansion sizes in an *srs2* mutant were also consistent with a replicational source. The helicase activity of Srs2 is important for preventing expansions in vivo (although the related helicase Sgs1 cannot compensate for loss of Srs2), and purified Srs2 is substantially faster than bacterial UvrD helicase at unwinding a DNA substrate that mimics a TNR hairpin. Together these results indicate a novel, specific, and direct role for Srs2 in preventing TNR expansions.

What molecular features might account for the selective inhibition of TNR expansions by Srs2? We suggest that Srs2 acts in vivo to unwind most TNR hairpins before they become fixed as expansions. The unwound hairpin might either reanneal in register with the template strand or become a substrate for nuclease cleavage and thereby remove the excess DNA prior to completion of the expansion process. This idea helps explain why *srs2* strains are mutators only at TNRs, which are unique among microsatellites in using a hairpin-based mechanism to expand in large increments. Other repeating elements such as dinucleotides change length primarily one or two repeats at a time, due to replication slippage (41), and thus the small loop-out intermediates would not present a target for Srs2 helicase action. To explain the stabilization of TNR expansions but not contractions, it is possible that Srs2 may not have access to hairpins on the template strand or that loading

of the helicase may be precluded. If true, contractions could occur regardless of Srs2 status.

The final issue is the TNR length dependence of the *srs2* phenotype. While both 13-repeat and 25-repeat tracts are protected by Srs2, a greater protective effect is seen for the shorter allele. Perhaps Srs2 helicase is more active on shorter hairpins, but longer hairpins are not unwound as readily. Helicase assays showed that longer times are required for Srs2 unwinding of 10-repeat versus 5-repeat substrates. Alternatively, the protective function of Srs2 may be reduced for the $(CTG)_{25}$ tract because it is already relatively unstable. These possibilities will be addressed in future work.

Our results also suggest that Srs2 helicase prevents expansions primarily in conjunction with DNA polymerase delta, since the *srs2K41A* mutant, the *pol32*Δ mutant, and the *srs2 pol32*Δ double mutant all gave high $(CTG)_{13}$ expansion rates within twofold of the *srs2* value. Strains bearing the *srs2* or *pol32*Δ mutation also showed a similar dependence on repeat tract length; for $(CTG)_{25}$, a smaller increase in expansion phenotype was seen for *srs2* (Table 1) and no significant effect was seen in *pol32*Δ strains (36). Polymerase delta is essential for replication, but it also acts during recombination and in some repair pathways (5). Recombination is not a major source of TNR instability in our system, as the loss of *RAD51* or *RAD52* functions in *srs2* backgrounds reduced the expansion rate by 3.5-fold or less compared to an *srs2* single mutant. Srs2 and polymerase delta must work primarily through replication and/or repair to inhibit expansions. A role for Srs2 in DNA replication has been suggested previously (21), and expansion sizes in an *srs2* mutant are consistent with replication-mediated events (12). We are not aware of any reports in the literature testing whether repeat replication is compromised in *srs2* mutants, but this would be a very interesting question to address.

Several models consistent with our epistasis results can be envisioned for inhibiting expansions. The first is a direct protein-protein interaction of Srs2 and polymerase delta. For example, the polymerase might stall when it synthesizes a TNR tract that subsequently folds into a hairpin and then recruit Srs2 for unwinding. A putative Srs2-polymerase delta complex might help explain the circa twofold reduction in expansion rates for *srs2K41A* compared to the *srs2* null mutant. This difference allows for the possibility that there is an ATPase-independent component for inhibiting expansions that can be conferred by the point mutant protein (perhaps via protein-protein contacts). Our viewpoint is that the large majority (17-fold) of the *srs2* effect is ATPase dependent. If a direct physical interaction between Srs2 and polymerase delta is necessary, however, our results with overexpression of Srs2 in a *pol32*Δ strain indicate that Pol32 cannot be the only crucial protein-protein contact (keeping in mind the caveat regarding overexpression).

A second model is that Srs2 and Pol32 inhibit expansions via the same pathway but without direct contact. Model two is in closer agreement with the Srs2 overexpression study. A third possibility is that expansions arise by a more indirect mechanism, such as defects in checkpoint response associated with loss of Srs2 (23, 46). If true, perhaps Srs2 is involved in a specialized repair process that responds to replicational stress (23), which in the case of triplet repeats could be replication fork stalling. Model three does not seem to be mutually exclusive with the first two models.

In *srs2 rad52* strains, the propensity to expand is similar to that of an *srs2* single mutant. Also, *rad51* and *rad52* single mutants show no specific phenotype in our assay. Both findings are consistent with our interpretation that recombination plays only a minor role in expansions in our system. In contrast, the *srs2 rad51* double mutant showed a larger (≈3.5-fold) reduction in expansion rates than the *srs2* strains, suggesting some specialized effect of Rad51. Perhaps Rad51 sequesters or inhibits another protein that can partially substitute for Srs2. In *rad51 srs2* mutants, this other factor might then act to avoid some expansions that would otherwise occur due to the absence of Srs2. The identity of this putative second factor is unknown. Alternatively, the absence of both Srs2 and Rad51 might result in alteration in repair pathway choice, leading to an effect on expansion rate. This alternate pathway remains hypothetical, however, until appropriate experiments can be done.

In summary, our results show that the Srs2 helicase acts through an important new mechanism to provide a selective and potent block to TNR expansions. These studies provide a paradigm for how one helicase, Srs2, can suppress expansions.

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REFERENCES

1. **Aboussekhra, A., R. Chanet, A. Adjiri, and F. Fabre.** 1992. Semidominant suppressors of Srs2 helicase mutations of *Saccharomyces cerevisiae* map in the *RAD51* gene, whose sequence predicts a protein with similarities to prokaryotic RecA proteins. Mol. Cell. Biol. **12:**3224–3234.
2. **Aboussekhra, A., R. Chanet, Z. Zgaga, C. Cassier-Chauvat, M. Heude, and F. Fabre.** 1989. *RADH*, a gene of *Saccharomyces cerevisiae* encoding a putative DNA helicase involved in DNA repair. Characteristics of *radH* mutants and sequence of the gene. Nucleic Acids Res. **17:**7211–7219.
3. **Aguilera, A., and H. L. Klein.** 1989. Genetic and molecular analysis of recombination events in *Saccharomyces cerevisiae* occurring in the presence of the hyper-recombination mutation. hpr1. Genetics **122:**503–517.
4. **Bhattacharyya, S., M. L. Rolfsmeier, M. J. Dixon, K. Wagoner, and R. S. Lahue.** 2002. Identification of *RTG2* as a modifier gene for CAG•CTG repeat instability in *Saccharomyces cerevisiae*. Genetics **162:**579–589.
5. **Burgers, P. M. J.** 1998. Eukaryotic DNA polymerases in DNA replication and DNA repair. Chromosoma **107:**218–227.
6. **Christianson, T. W., R. S. Sikorski, M. Dante, J. H. Shero, and P. Hieter.** 1992. Multifunctional yeast high-copy-number shuttle vectors. Gene **110:** 119–122.
7. **Cleary, J. D., and C. E. Pearson.** 2003. The contribution of CIS-elements to disease-associated repeat instability: clinical and experimental evidence. Cytogenet. Genome Res. **100:**25–55.
8. **Cummings, C. J., and H. Y. Zoghbi.** 2000. Fourteen and counting: unraveling trinucleotide repeat diseases. Hum. Mol. Genet. **9:**909–916.
9. **Dong, Z., and M. Fasullo.** 2003. Multiple recombination pathways for sister chromatid exchange in *Saccharomyces cerevisiae*: role of *RAD1* and the *RAD52* epistasis group genes. Nucleic Acids Res. **31:**2576–2585.
10. **Freudenreich, C. H., S. M. Kantrow, and V. A. Zakian.** 1998. Expansion and length-dependent fragility of CTG repeats in yeast. Science **279:**853–856.
11. **Gacy, A. M., G. Goellner, N. Juranic, S. Macura, and C. T. McMurray.** 1995. Trinucleotide repeats that expand in human disease form hairpin structure in vitro. Cell **81:**533–540.
12. **Gordenin, D. A., T. A. Kunkel, and M. A. Resnick.** 1997. Repeat expansion—all in a flap? Nat. Genet. **16:**116–118.

13. **Hegde, V., and H. Klein.** 2000. Requirement for the *SRS2* DNA helicase gene in non-homologous end joining in yeast. Nucleic Acids Res. **28:**2779–2783.
14. **Henderson, S. T., and T. D. Petes.** 1992. Instability of simple sequence DNA in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **12:**2749–2757.
15. **Huang, M.-E., A. de Calignon, A. Nicolas, and F. Galibert.** 2000. *POL32*, a subunit of the *Saccharomyces cerevisiae* DNA polymerase delta, defines a link between DNA replication and the mutagenic bypass repair pathway. Curr. Genet. **38:**178–187.
16. **Klein, H.** 2000. A radical solution to death. Nat. Genet. **25:**132–134.
17. **Kovtun, I., G. Goellner, and C. T. McMurray.** 2001. Structural features of trinucleotide repeats associated with DNA expansion. Biochem. Cell Biol. **79:**325–336.
18. **Kramer, B., W. Kramer, M. S. Williamson, and S. Fogel.** 1989. Heteroduplex DNA correction in *Saccharomyces cerevisiae* is mismatch specific and requires functional *PMS* genes. Mol. Cell. Biol. **9:**4432–4440.
19. **Krejci, L., S. Van Komen, Y. Li, J. Villemain, M. S. Reddy, H. Klein, T. Ellenberger, and P. Sung.** 2003. DNA helicase Srs2 disrupts the Rad51 presynaptic filament. Nature **423:**305–309.
20. **Lea, D. E., and C. A. Coulson.** 1948. The distribution of the number of mutants in bacterial populations. J. Genet. **49:**264–284.
21. **Lee, S. K., R. E. Johnson, S. L. Yu, L. Prakash, and S. Prakash.** 1999. Requirement of yeast *SGS1* and *SRS2* genes for replication and transcription. Science **286:**2339–2342.
22. **Leeflang, E. P., S. Tavare, P. Marjoram, C. O. S. Neal, J. Srinidhi, M. E. MacDonald, M. de Young, N. S. Wexler, J. F. Gusella, and N. Arnheim.** 1999. Analysis of germline mutation spectra at the Huntington's disease locus supports a mitotic mutation mechanism. Hum. Mol. Genet. **8:**173–183.
23. **Liberi, G., I. Chiolo, A. Pellicioli, M. Lopes, P. Plevani, M. Muzi-Falconi, and M. Foiani.** 2000. Srs2 DNA helicase is involved in checkpoint response and its regulation requires a functional Mec1-dependent pathway and Cdk1 activity. EMBO J. **19:**5027–5038.
24. **Liu, Y., and R. A. Bambara.** 2003. Analysis of human flap endonuclease 1 mutants reveals a mechanism to prevent triplet repeat expansion. J. Biol. Chem. **278:**3278–13739.
25. **Liu, Y., H. Zhang, J. Veeraraghavan, R. A. Bambara, and C. H. Freudenreich.** 2004. *Saccharomyces cerevisiae* flap endonuclease I uses flap equilibration to maintain triplet repeat stability. Mol. Cell. Biol. **24:**4049–4064.
26. **Manivasakam, P., S. C. Weber, J. McElver, and R. H. Schiestl.** 1995. Microhomology mediated PCR targeting in *Saccharomyces cerevisiae*. Nucleic Acids Res. **23:**2799–2800.
27. **Mankouri, H. W., T. J. Craig, and A. Morgan.** 2002. *SGS1* is a multicopy suppressor of *srs2*: functional overlap between DNA helicases. Nucleic Acids Res. **30:**1103–1113.
28. **Matson, S. W.** 1986. *Escherichia coli* helicase II (*uvrD* gene product) translocates unidirectionally in a 3′ to 5′ direction. J. Biol. Chem. **261:**10169–10175.
29. **Matson, S. W., and K. A. Kaiser-Rogers.** 1990. DNA helicases. Annu. Rev. Biochem. **59:**289–329.
30. **Mechanic, L. E., Frankel, B. A., and Matson, S. W.** 2000. Escherichia coli MutL loads DNA helicase II onto DNA. J. Biol. Chem. **275:**38337–38346.
31. **Mechanic, L. E., Frankel, B. A., and Matson, S. W., M. C. Hall, and S. W. Matson.** 1999. Escherichia coli DNA helicase II is active as a monomer. J. Biol. Chem. **274:**12488–12498.
32. **Mechanic, L. E., M. E. Latta, and S. W. Matson.** 1999. A region near the C-terminal end of *Escherichia coli* DNA helicase II is required for single-stranded DNA binding. J. Bacteriol. **181:**2519–2526.
33. **Miret, J. J., L. Pessoa-Brandao, and R. S. Lahue.** 1998. Orientation-dependent and sequence-specific expansions of CTG/CAG trinucleotide repeats in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **95:**12438–12443.
34. **Myung, K., A. Datta, C. Chen, and R. D. Kolodner.** 2001. SGS1, the *Saccharomyces cerevisiae* homologue of BLM and WRN, suppresses genome instability and homeologous recombination. Nat. Genet. **27:**113–116.
35. **Paulson, H. L., and K. H. Fischbeck.** 1996. Trinucleotide repeats in neurogenetic disorders. Annu. Rev. Neurosci. **19:**79–107.
36. **Pelletier, R., A. S. Krasilnikova, G. M. Samadishwily, R. S. Lahue, and S. M. Mirkin.** 2003. Replication and expansion of trinucleotide repeats in yeast. Mol. Cell. Biol. **23:**1349–1357.
37. **Rolfsmeier, M. L., M. J. Dixon, L. Pessoa-Brandao, R. Pelletier, J. J. Miret, and R. S. Lahue.** 2001. Cis-elements governing trinucleotide repeat instability in *Saccharomyces cerevisiae*. Genetics **157:**1569–1579.
38. **Rolfsmeier, M. L., and R. S. Lahue.** 2000. Stabilizing effects of interruptions on trinucleotide repeat expansions in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **20:**173–180.
39. **Rose, M. D., F. Winston, and P. Hieter.** 1988. Methods in yeast genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
40. **Schweitzer, J. K., and D. M. Livingston.** 1998. Expansions of CAG repeat tracts are frequent in a yeast mutant defective in Okazaki fragment maturation. Hum. Mol. Genet. **7:**69–74.
41. **Sia, E. A., R. J. Kokoska, M. Dominska, P. Greenwell, and T. D. Petes.** 1997. Microsatellite instability in yeast: dependence on repeat unit size and DNA mismatch repair genes. Mol. Cell. Biol. **17:**2851–2858.
42. **Sikorski, R. S., and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:**19–27.
43. **Spiro, C., and C. T. McMurray.** 2003. Nuclease-deficient FEN-1 blocks Rad51/BRCA1-mediated repair and causes trinucleotide repeat instability. Mol. Cell. Biol. **23:**6063–6074.
44. **Spiro, C., R. Pelletier, M. L. Rolfsmeier, M. J. Dixon, R. S. Lahue, G. Gupta, M. S. Park, X. Chen, S. V. S. Mariappan, and C. T. McMurray.** 1999. Inhibition of FEN-1 processing by DNA secondary structure at trinucleotide repeats. Mol. Cell **4:**1079–1085.
45. **Usdin, K., and E. Grabczyk.** 2000. DNA repeat expansions and human disease. Cell. Mol. Life Sci. **57:**914–931.
46. **Vaze, M. B., A. Pellicioli, S. E. Lee, G. Ira, G. Liberi, A. Arbel-Eden, M. Foiani, and J. E. Haber.** 2002. Recovery from checkpoint-mediated arrest after repair of a double-strand break requires Srs2 helicase. Mol. Cell **10:**373–385.
47. **Veaute, X., J. Jeusset, C. Soustelle, S. C. Kowalczykowski, E. Le Cam, and F. Fabre.** 2003. The Srs2 helicase prevents recombination by disrupting Rad51 nucleoprotein filaments. Nature **423:**309–312.
48. **White, P. J., R. H. Borts, and M. C. Hirst.** 1999. Stability of the human fragile X $(CGG)_n$ triplet repeat array in *Saccharomyces cerevisiae* deficient in aspects of DNA metabolism. Mol. Cell. Biol. **19:**5675–5684.