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Orientation Dependence of Trinucleotide CAG Repeat Instability in *Saccharomyces cerevisiae*

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To examine the chromosomal stability of repetitions of the trinucleotide CAG, we have cloned CAG repeat tracts onto the 3′ end of the *Saccharomyces cerevisiae ADE2* gene and placed the appended gene into the *ARO2* locus of chromosome VII. Examination of chromosomal DNA from sibling colonies arising from clonal expansion of strains harboring repeat tracts showed that repeat tracts often change in length. Most changes in tract length are decreases, but rare increases also occur. Longer tracts are more unstable than smaller tracts. The most unstable tracts, of 80 to 90 repeats, undergo changes at rates as high as 3×10^{-2} changes per cell per generation. To examine whether repeat orientation or adjacent sequences alter repeat stability, we constructed strains with repeat tracts in both orientations, either with or without sequences 5′ to *ADE2* harboring an autonomously replicating sequence (ARS; replication origin). When CAG is in the *ADE2* coding strand of strains harboring the ARS, the repeat tract is relatively stable regardless of the orientation of *ADE2*. When CTG is in the *ADE2* coding strand of strains harboring the ARS, the repeat tract is relatively unstable regardless of the orientation of *ADE2*. Removal of the ARS as well as other sequences adjacent to the 5′ end of *ADE2* alters the orientation dependence such that stability now depends on the orientation of *ADE2* in the chromosome. These results suggest that the proximity of an ARS or another sequence has a profound effect on repeat stability.

Expansions of repetitions of the trinucleotide CAG are the cause of a number of human inherited, dominant neurological and neuromuscular diseases, including Huntington's disease (14), two forms of spinocerebellar ataxia (type 1 and Machado-Joseph disease) (17, 20), and myotonic dystrophy (3, 7, 19). Although CAG trinucleotide repetitions are present in normal alleles of the genes giving rise to these disorders, mutant alleles have tracts which are longer than those within the normal range. The long tracts within disease alleles are unstable in that children often inherit a repeat length different from that found in their affected parent. The instability most likely reflects replicative errors which occur either during the meiotic division of gametogenesis or during the mitotic divisions preceding it.

The underlying cause of the instability is thought to reflect the ability of CAG repeats to form palindrome-like structures (8, 22). Such structures may present problems to the replication fork as it passes through them. One study using small CAG repeats embedded in palindromes carried on phage lambda showed that they were inhibitory to plaque formation (6). Studies with *Escherichia coli* have also shown that CAG repeats undergo both contractions and expansions when propagated in a bacterial plasmid (16).

We decided to examine the stability of CAG repeats in *Saccharomyces cerevisiae* because the chromatin structure and chromosomal replication of this simple eukaryote have many similarities to the chromosomal mechanics of more complex eukaryotes. Examination of CAG repeat stability in *S. cerevisiae* affords controlled opportunities to understand why this sequence exhibits both organism-specific and gene-specific behavior in its stability. For example, transgenic mice carrying human disease genes do not exhibit instability of CAG repeat tracts (2, 4). In humans, except for very long CAG tracts (>1,000 repeat units), repeats are mitotically stable but meiotically unstable, often showing sex differences as well. Furthermore, the instability of CAG repeats in human genes differs among those which contain this sequence. Judged by the difference in tract length between parent and child, some genes (those related to Huntington's disease and spinocerebellar ataxia type 1) show a seemingly continual small, incremental average increase, some (related to Machado-Joseph disease) show both small increases and contractions, while some (related to myotonic dystrophy) undergo very large (>100-repeat-unit) expansions.

The issue at hand is to learn how yeast cells can be used to model and understand these organismal and gene-specific differences. In this report we show that CAG repeat tracts are unstable in an orientation-specific manner when placed in a yeast chromosome.

MATERIALS AND METHODS

Yeast strain and growth. The yeast strain SSL204a (MATa *ade2 ura3 trp1 leu2 his3*) (1) was used for these experiments and was cultured by standard procedures at 30°C.

Cloning of CAG repeats. We used the KS+ vector (Stratagene) and *E. coli* JA300 (5) or stbl2 (Life Technologies) for all the preparatory steps. A clone of the human ataxin-1 gene that contained an uninterrupted repeat of 82 units [$(CAG)_{82}$] was obtained from Harry Orr (University of Minnesota) (20). PCR primers were designed to extend from the unique sequences of ataxin-1 flanking its CAG repeat tract into the beginning of the repeated sequence. These also included an *Nhe*I recognition sequence at their 5′ ends. The PCR product was placed into the yeast *ADE2* gene at a *Hin*dIII site at the extreme 3′ end of the coding region which had been filled in to create an *Nhe*I site (23). DNA sequencing was used to orient the clones.

Two *ADE2* fragments containing different amounts of flanking sequences were used in our studies. Initially we used a 3.6-kb genomic fragment in which the 1,713-bp *ADE2* sequence lies mostly within the 3′ half of the fragment. We also used PCR primers to prepare a smaller, 2.2-kb fragment containing *ADE2*. This smaller fragment had been shown by Stotz and Linder (23) to retain *ADE2* expression but lacks the autonomously replicating sequence (ARS) activity associated with sequences 5′ to *ADE2*.

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Placement of *ADE2* within the *ARO2* locus on chromosome VII. Both the 3.6- and the 2.2-kb *ADE2* sequences we used contained artificial *Bam*HI recognition sequences at their ends. These *Bam*HI fragments were placed in the unique *Bcl*I site within *ARO2* (15). The entire sequence was removed from the cloning vector and used to transform strain SSL204a to adenine prototrophy. Because the primers used to clone the repeats were designed to place them in frame within *ADE2* in either orientation and because the amino acids at the C terminus are apparently not needed for *ADE2* function, the interrupted copies of *ADE2* are functional. Ade^+ transformants were checked for phenylalanine and tyrosine auxotrophy (*aro2*). Southern blotting was also used to ensure the presence of a single copy of the *aro2*::*ADE2*$(CAG)_n$ disruption.

Propagation, DNA extraction, and PCR conditions. Transformant colonies were streaked onto yeast extract-peptone-dextrose agar to yield individual colonies. Entire colonies were removed from the agar and suspended in 1 ml of sterile water. A sample was removed, diluted, and plated on yeast extract-peptone-dextrose agar to yield approximately 200 colonies. DNA was extracted from the remainder of the sample with a Puregene kit (Gentra Systems) by using one-quarter of the volume recommended by the manufacturer for a 1-ml yeast culture. One-fifteenth of the DNA was used as a PCR template. Besides the template DNA, the PCR mixture included 1× cloned *Pfu* buffer (Stratagene), 250 μM each deoxynucleoside triphosphate, 1 μM each of the primers DMLAde2b (5′-ATTTGCTGTACAAGTATATCAATAAAC-3′) and DMLAde2c (5′-GTTAGAAACTGTCGGTTACGAAGC-3′), and 1 U of cloned *Pfu* polymerase (Stratagene). These primers are complementary to the yeast *ADE2* gene, and primer DMLAde2c extends into the filled-in *Hin*dIII site. Primer DMLAde2c was end-labeled with [γ-^{32}P]ATP by using polynucleotide kinase (New England BioLabs) as described by the manufacturer. PCR conditions were 94°C for 4 min; 30 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min; and one extra minute of extension at 72°C. The products were run on standard 5% polyacrylamide-urea sequencing gels. *Hpa*II-cut KS+ vector (Stratagene) filled in with [α-^{32}P]dCTP was used as a size standard. After electrophoresis the gel was dried and exposed to a storage phosphor screen (Molecular Dynamics).

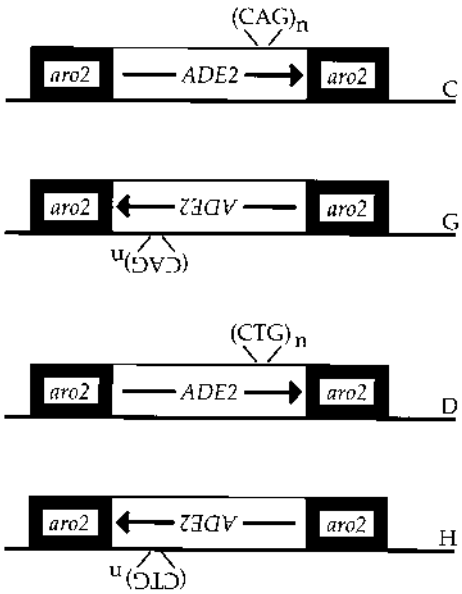

FIG. 1. Placement of CAG trinucleotide in yeast chromosome VII. The organization of the repeat tracts in *ADE2* and its disruption in *ARO2* on chromosome VII are shown. The strains are designated by letters placed to the right on the line representing chromosome VII. Eight strains were created, four with a 3.6-kb and four with a 2.2-kb *ADE2* fragment. The strains with the 3.6-kb *ADE2* fragment are shown here; the corresponding strains with the smaller, 2.2-kb *ADE2* fragment are designated C−, G−, D−, and H−.

RESULTS

Placement of repeat sequences in chromosome VII. To investigate the stability of trinucleotide CAG repeats, repeat tracts were placed within yeast chromosome VII. The repeat tracts were obtained from the human ataxin-1 gene by copying them by PCR (20). The repeat tracts were cloned into the C-terminal sequence of the yeast *ADE2* gene so that either CAG or its complement CTG appeared in the coding strand (Fig. 1). These interruptions do not incapacitate *ADE2*. In turn, copies of the *ADE2* gene with the repeat tracts were used to disrupt a cloned copy of *ARO2*. The disrupted *ARO2* sequence was then used to replace the wild-type copy on chromosome VII by selecting for Ade^+. Eight such strains with uninterrupted CAG tracts copied from a human allele with 82 repeat units were constructed with either CAG or CTG in the *ADE2* coding strand, with each of the two possible orientations of *ADE2* and with or without the sequences that lie adjacent to the 5′ end of *ADE2* (Fig. 1). Because of the inherent instability of this sequence in the cloning host, *Escherichia coli* (16), and in *S. cerevisiae*, the primary strains did not necessarily contain 82 repeat units.

Analysis of repeat length changes. Our initial studies were done on the four strains with the 3.6-kb *ADE2* fragment containing the adjacent 5′ sequences. We assayed for changes in repeat tracts by extracting DNA from individual colonies. Using primers complementary to *ADE2* sequences flanking the repeats, we carried out PCR to measure the length of the repeat tracts. The PCR products were displayed on a DNA sequencing gel (Fig. 2 through 4).

Most PCRs yielded two major products. The smaller was a fragment 77 bases in length derived from the *ade2* copy on chromosome XV. This served as a positive control for the PCRs. The second product was longer and derived from the repeat tract within the *ADE2* sequence disrupting *ARO2* on chromosome VII (Fig. 2 through 4).

Inspection of individual lanes reveals a number of confounding bands (Fig. 2 and 3). We believe that these arise by two different mechanisms. First, PCRs of trinucleotide repeats often produce stuttering by three bases. Our PCR conditions produced a ladder of faint bands descending from the bands of the long repeat tracts. That this ladder most likely represents a PCR artifact rather than a population of cells with different tract lengths can be shown by cloning individual cells from a strain containing a stable repeat tract (Fig. 3) and observing that each cell contains a band corresponding to the long repeat tract plus the descending ladder of bands. Second, faint shadow bands sometimes appear above and below the repeat band. Most of these are presumed to be electrophoresis artifacts, as they usually appear at a fixed distance above or below the major bands and often appear at an angle different from that of the repeat band.

For these analyses a single colony containing an uninterrupted repeat was chosen and was characterized for its CAG repeat tract length in order to determine the original size of the tract. A portion of the colony was also dispersed, and individual cells were permitted to grow into colonies. DNA was extracted from these sibling colonies, and their CAG tract lengths were determined and compared to the original size. An example of such an analysis is shown in Fig. 2. Of the 15 sibling colonies, 4 no longer contain the original repeat tract band; instead, they contain a major band of smaller size (Fig. 2, lanes a, d, e, and n). We interpret these as representing colonies arising from cells within the original colony which had undergone a change prior to the original colony's dispersal into individual cells. In support of this interpretation, we dispersed the cells from these sibling colonies and found that the colonies arising from such cells contained tracts of the new length. All of the changes appear to be reductions in size by multiples of three bases because the new, smaller bands coincide with the faint ladder of bands descending from larger bands in adjacent lanes. Most are not small changes (one or two repeats) but larger changes (five or more repeats). For example, the average change for the 16 examples found for strain D with 78

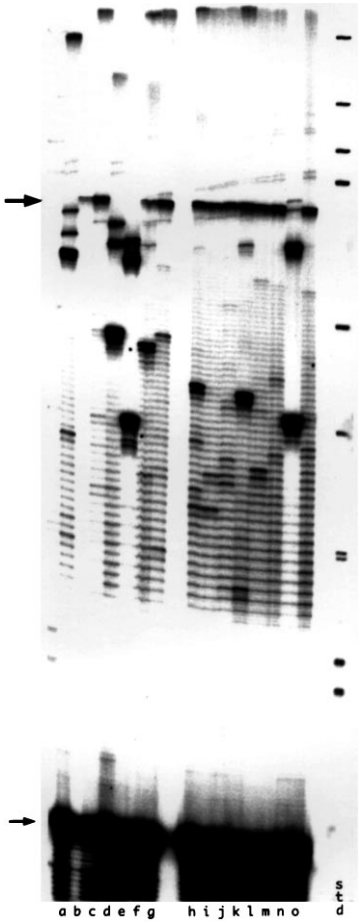

FIG. 2. Unstable repeat tracts. PCR products from DNA isolated from 15 sibling colonies (lanes a through o) originating from a colony of strain D with 78 repeats are shown. The thin arrow (bottom) marks the position of the PCR product 77 bases in length derived from the *ADE2* locus on chromosome XV, and the bold arrow (top) points to the position of the original-size band containing the CAG repeat tract. The size of the original band was previously determined by assaying DNA from the colony giving rise to the sibling colonies in this analysis. (We believe that the faint band present at the position of the original band in lane n is an electrophoresis artifact as described in Results because it appears to be part of the continuum of faint artifact bands seen in lanes g through n that have a cant different from the slope of the original-size bands.) The standard lane (std) contains ^{32}P-labeled *Hpa*II-digested KS+.

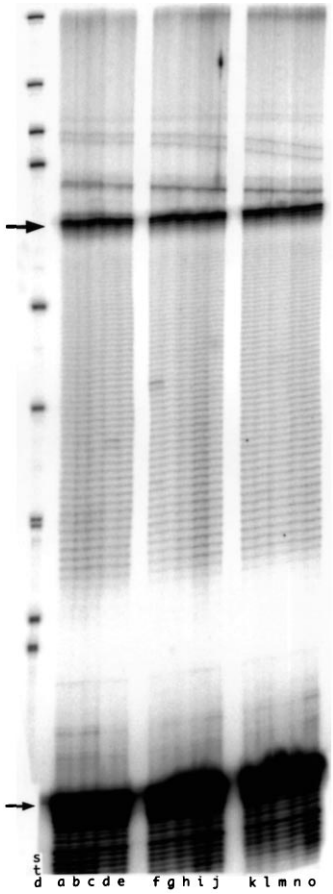

FIG. 3. Stable repeat tracts. PCR products from DNA isolated from 15 sibling colonies (lanes a through o) originating from a colony of strain G with 65 repeats are shown. The standard lane (std) and arrows are explained in the legend to Fig. 2.

repeats (Fig. 2 and Table 1) is a decrease of 44 repeat units (60, 60, 58, 53, 52, 50, 50, 48, 40, 40, 38, 38, 38, 28, 23, and 22 repeat units). This result reveals the instability of such long, uninterrupted repeats in *S. cerevisiae*. Of the four strains in our initial studies, two proved to be relatively unstable (D and H) and two proved to be relatively stable (C and G), as described in more detail below.

Instability increases with tract length. To investigate the dependence of stability on tract length, we took advantage of the inherent instability of the repeat tracts to produce new strains with shorter tract lengths. Analyses of these new strains revealed that as the unstable repeat tracts became smaller, they also became more stable (Table 1 and Fig. 5). By the time they reached a size of approximately 30 to 40 repeat units, we did not find changes among the 30 sibling colonies we sampled for each isolate.

Increases in tract length are rare. Although a majority of the changes occurred with a reduction in tract length, we also found four examples of increases among the initial set of four strains containing the longer *ADE2* fragment (Table 1). Unlike the numerous decreases, which were often large, the four examples of increases were relatively small. Their average size was an increase in seven units (30→32, 60→70, 65→69, 72→87). An example is shown in Fig. 4. Furthermore, two of the four increases occurred in the more stable strains (G and C). Of the three changes that we observed in strains G and C, two were increases in tract length and only one was a decrease. In contrast, for the more unstable strains (H and D), only 2 of the 41 changes were increases. Thus, increases in tract length are infrequent and can occur when either CAG or CTG is in the *ADE2* coding strand.

Estimating the rate of instability. An estimate of the rate at which changes occur can be made from the frequency of colonies which are missing the band representing the original tract length and have a new (smaller or bigger) band (Table 1). An upper estimate was made for strains D and H, which have change frequencies as high as 0.3. Calculations according to the method of Lea and Coulson (18), using 0.3 as a median value for the mutation frequency and a colony size of 5×10^6 cells, yield a rate of approximately 3×10^{-2} changes per cell per generation.

Repeat tract instability depends on CAG tract orientation. Initially, we characterized the four strains that contained the larger (3.6-kb) *ADE2* fragment. Of the four strains with uninterrupted repeats, two were unstable and two were relatively

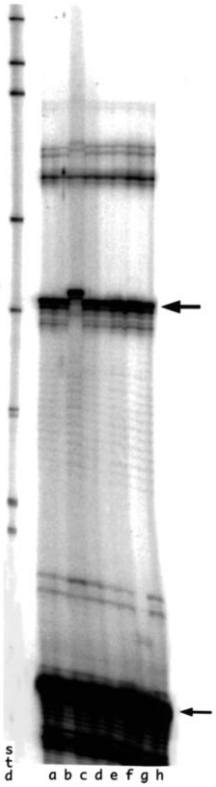

FIG. 4. Example of an increase in tract length. PCR products from DNA isolated from eight sibling colonies (lanes a through h) originating from a colony of strain C with 30 repeats are shown. The standard lane (std) and arrows are explained in the legend to Fig. 2. Lane c shows an increase of two repeat units.

TABLE 1. CAG repeat changes

Strain	Trinucleotide and no. of repeats	No. of sibling colonies			
		Total	With original-size band	Without original band	
				With smaller band	With larger band
G	$(CAG)_{69}$	30	30	0	0
G	$(CAG)_{65}$	30	29	0	1
G	$(CAG)_{30}$	30	30	0	0
C	$(CAG)_{82}$	27	27	0	0
C	$(CAG)_{82}$	29	28	1	0
C	$(CAG)_{42}$	25	25	0	0
C	$(CAG)_{30}$	30	29	0	1
H	$(CTG)_{90}$	29	23	6	0
H	$(CTG)_{70}$	30	20	9	1
H	$(CTG)_{52}$	30	28	2	0
H	$(CTG)_{32}$	30	30	0	0
D	$(CTG)_{78}$	29	21	8	0
D	$(CTG)_{78}$	29	21	8	0
D	$(CTG)_{60}$	29	24	4	1
D	$(CTG)_{55}$	30	28	2	0
D	$(CTG)_{40}$	30	30	0	0
D	$(CTG)_{36}$	30	30	0	0
D	$(CTG)_{26}$	30	30	0	0
D	$(CTG)_{20}$	30	30	0	0

stable (Table 1 and Fig. 5). In the two more stable strains the repeat tract was oriented with CAG in the *ADE2* coding strand, whereas in the two unstable strains CTG appeared in the *ADE2* coding strand. Because stability does not depend on the orientation of the *ADE2* disruption in *ARO2*, the elements which control stability most likely reside in the 3.6-kb *ADE2* fragment and not in the surrounding sequences of chromosome VII.

The difference in stability is also seen in another banding pattern. In this class the original band is not lost but is joined by a smaller (or larger) band (Fig. 2, lanes f, h, and k). We interpret these as representing colonies in which the tract changed in length in one of the first few generations after dispersal into individual cells. In support of this interpretation, we have dispersed cells from such colonies to propagate new colonies and found that DNA from the new colonies contains either the original-size tract or a tract the size of the smaller (or larger) band. Inspection of unstable tracts like those in Fig. 2 reveals one or more bands which reinforce the faint ladder descending from the original band in almost all lanes. These reinforcing bands are much rarer, and often fainter, in displays of stable tracts such as those shown in Fig. 3 and 4. An example of one such reinforcing band is seen in Fig. 3, lane f. Thus, the prominence of reinforcing bands is another indication of the instability resulting from orientation.

Investigation of the orientation dependence. The orientation independence of the repeat stability suggests that the responsible sequence elements are present within the 3.6-kb fragment containing *ADE2*. One possible cause might be the ARS that resides 5′ to *ADE2* (21, 23). If this ARS is an actual replication origin, then instability correlates with CTG being the lagging-strand template.

To investigate this possibility, four strains (C−, D−, G−, and H−) that eliminate the 5′ sequences responsible for the ARS activity yet maintain *ADE2* transcription were constructed (23). The model shows that elimination of the ARS adjacent to *ADE2* places the repeats in a replication fork created by the replication origin that is normally responsible for replication of the *ARO2* region of chromosome VII (Fig. 6). Although there is no a priori knowledge of the position of the flanking replication origin on chromosome VII, this model predicts that one of the relatively unstable clones will become more stable, while one of the relatively stable clones will become more unstable (Fig. 6). Furthermore, the model demands consistency in the orientation of CTG and CAG with respect to relative instability and stability.

Analyses of tract instability for the four strains lacking the

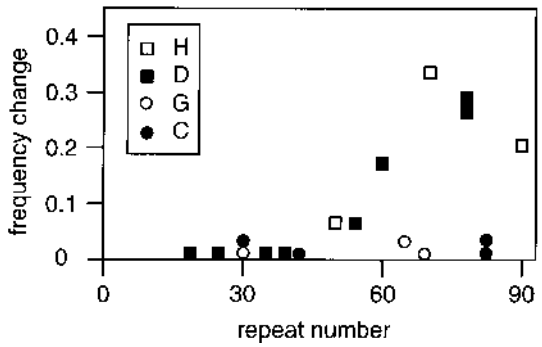

FIG. 5. Relative stability of repeat tracts. The results of all analyses (Table 1) are plotted to show the relationship between repeat tract size and instability. Both increases and decreases are included in the plotted values. When two values are given for a particular strain and tract length, these represent independent trials.

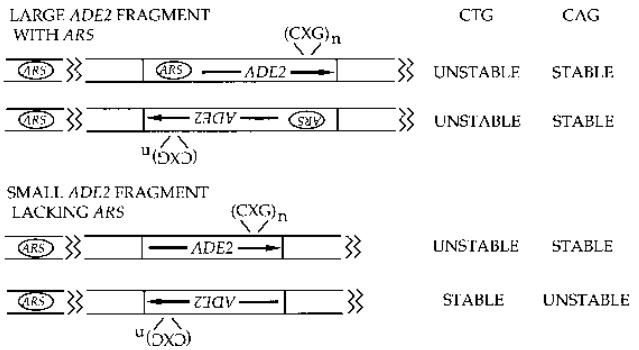

FIG. 6. Orientation dependence of instability. The diagram shows the stabilities of repeat tracts predicted by the hypothesis that an ARS controls stability. By this hypothesis tract stability is independent of *ADE2* orientation when the *ADE2* clone contains an ARS (strains C, G, D, and H). When this ARS is removed, stability depends on the ARS that is normally responsible for replication through *ARO2* on chromosome VII, and stability becomes dependent on *ADE2* orientation. The placement of the ARS on chromosome VII to the left is consistent with the data.

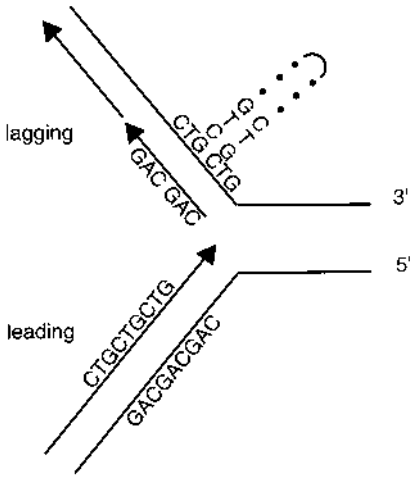

FIG. 7. Deletion of repeat tract units by replication. The diagram shows the formation of a foldback structure when CTG is the lagging-strand template and replication through this structure to yield a deletion in the Okazaki fragment.

ARS adjacent to *ADE2* shows that the relative stability has shifted in a pattern expected if orientation with respect to a replication origin is responsible for the repeat instability (Table 2). Most notably, the repeat tract in strain H− shows no instability, whereas the tract in strain H is unstable. Furthermore, the tract in strain G− is more unstable than the relatively stable tract in strain G. (The value for H− was compared with the combined values for strain H with 70 and 52 repeat units and yielded a chi-square value of 11.2. Similarly, the value for G− was compared with the values for strain G with 69 and 65 repeat units and yielded a chi-square value of 7.68.) Strains C− and D− retain their relative stability and instability, respectively. (Chi-square values for C and C− and for D and D− are 0.457 and 0.955, respectively.) Assuming that placement of CTG as the lagging-strand template creates instability, the relative stabilities of strains C−, D−, G−, and H− place the responsible replication origin 5′ to *ARO2*.

DISCUSSION

CAG repeat tract instability in *S. cerevisiae* has some similarities with and unique differences from tract instability in other organisms. As in other organisms, CAG repeat tracts are inherently unstable in *S. cerevisiae*. Furthermore, repeat tracts can either expand or contract. A unique difference between *S. cerevisiae* and other organisms is the predominance of large deletions coupled with rarer, smaller expansions. Although such large quantal decreases are observed in *E. coli*, large increases are not as infrequent in *E. coli* as they are in *S. cerevisiae* (16).

The most striking feature of CAG repeat instability in *S. cerevisiae* is the effect that repeat orientation and adjacent sequences have on stability. Initially, we observed that when CAG appears in the *ADE2* coding strand, long tracts are relatively stable, whereas when CTG appears in the *ADE2* coding strand, the repeat is relatively unstable. This relative difference occurs regardless of the orientation of *ADE2* in the chromosome. These initial results suggested that a sequence within the 3.6-kb fragment containing *ADE2* was responsible for the relative difference. One sequence that might affect stability is the ARS at the 5′ end of the *ADE2* coding sequence (21, 23). Elimination of this ARS element, as well as other sequences adjacent to *ADE2*, altered the relative stability of repeat tracts such that one of the two unstable tracts became more stable and one of the two stable tracts became more unstable (Table 2). These changes were consistent with placement of a replication origin (or another controlling sequence) 5′ to the *ARO2* locus on chromosome VII (Fig. 6). Thus, our working hypothesis is that the proximity of a replication origin is responsible for the asymmetry in stability between CTG and CAG within the *ADE2* coding strand. If the replication origin hypothesis is correct, this would mean that the repeat is much less stable when CTG is the lagging-strand template. Furthermore, when CTG is the lagging-strand template, contractions are far more likely than expansions (Fig. 7). When CAG is the lagging-strand template, small expansions are as prevalent as contractions. Thus, the behavior of CAG repeats in *S. cerevisiae* represents an extreme of a phenomenon observed in *E. coli*, i.e., a predominance of expansions when CAG is the lagging-strand template and a predominance of contractions when CTG is the lagging-strand template (16). We note that positioning with respect to replication origins may be one factor in the differences in behavior of CAG repeat tracts among the human disease genes.

The behavior of trinucleotide CAG repeats in *S. cerevisiae* is different from the behavior of GT dinucleotide repeats in the same organism. The dinucleotide repeats undergo mostly small changes (one or two units) (11, 12, 24). Although biases in the ratio of increases to decreases also occur in GT dinucleotide repeat changes, the biases are difficult to interpret because of the reading frame selection imposed on the experimental systems. More importantly, no apparent effect was observed by reversing the orientation of the GT dinucleotide repeat with

TABLE 2. CAG repeat changes in the 2.2-kb *ADE2* clones

Strain	Trinucleotide and no. of repeats	No. of sibling colonies			
		Total	With original-size band	Without original band	
				With smaller band	With larger band
G−	$(CAG)_{75}$	59	48	10	1
C−	$(CAG)_{65}$	59	58	1	0
H−	$(CTG)_{60}$	60	60	0	0
D−	$(CTG)_{90}$	60	49	11	0

respect to a plasmid-borne ARS (13). The frequency of dinucleotide changes is responsive to genes which control mismatch repair (24). Because of the difference in behavior of di- and trinucleotide repeats, we suspect that the mismatch repair system may not be as important in controlling the fate of the trinucleotide repeats as it is in dinucleotide stability. Rather, the behavior of trinucleotide repeats is much more reminiscent of the behavior of palindromes whose deletions are influenced by replicating DNA polymerases (9, 10, 13, 25). We are currently examining the genetic control of trinucleotide stability.

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