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Replisome stalling and stabilization at CGG repeats that are responsible for chromosomal fragility

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Abstract

Expanded CGG repeats cause chromosomal fragility and hereditary neurological disorders in humans. Replication forks stall at CGG repeats in a length-dependent manner in primate cells and in yeast. Yeast Tof1 and Mrc1 proteins facilitate replication fork progression through CGG repeats. Remarkably, the fork-stabilizing role of Mrc1 does not involve its checkpoint function. Thus, chromosomal fragility might occur when forks stalled at expanded CGG repeats escape the S-phase checkpoint.

Fragile sites are chromosomal loci that look constricted or even broken upon replication inhibition. Rare fragile sites are associated with hereditary neurological disorders in humans, such as fragile X syndrome and FRAXE mental retardation, and are caused by expansions of (CGG)_n·(CCG)_n repeats². Both repeat expansions and chromosomal fragility seem to depend on faulty DNA replication caused by unusual structures of the repeats³. CGG repeats adopt hairpin-like and quadruplex-like DNA structures^{4,5}, which arrest DNA replication *in vitro*^{6,7} and in unicellular organisms^{8,9}. Is the same true for mammalian cells?

We monitored replication fork progression through CGG repeats in *COS-1* fibroblasts using a pSV2neo episome in COS-1 fibroblasts as previously described¹⁰. This vector contains a SV40 replication origin functional in cells expressing T-antigen. T-antigen acts as an initiator and a replicative DNA-helicase; the other components of the replisome come from the host cell. (CGG)_n repeats of normal (n=18), expansion threshold (n=40) or premutation (n=105) lengths were cloned into the pSV2neo vector either into a non-transcribed area or transcribed area. Each repeat was cloned in two orientations relative to the replication origin, positioning either (CGG)_n or (CCG)_n runs into the lagging strand template. Their distance from the origin was sufficient to separate stalled intermediates from the un-replicated molecules, avoiding problems in detection of repeat-mediated replication stalls¹¹.

Normal length CGG repeats did not affect replication fork progression in mammalian cells (Fig. 1). CGG repeats of the expansion threshold size caused defined replication stall signals

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AUTHOR CONTRIBUTIONS

I.V. designed and performed experiments in yeast and mammalian cells, and wrote a paper, C.F.S. performed replication studies in mammalian cells, A.A.S. performed cassettes for yeast knockouts, M.M.K. contributed to plasmid construction, S.M.M. designed experiments, supervised the whole project and wrote the paper.

whether they were placed in a transcribed or a non-transcribed area. The replication fork stalling further intensified for 105 CGG repeats. (A diffused descending arm of the Y arc here results from contractions of long repeats during plasmid propagation in *E. coli*.) Besides being stronger (Fig. 1b), the area of delayed replication spanned a large portion of the Y arc (Fig. 1a). In contrast, replication through a twice longer inverted repeat in mammalian cells produced a defined stall site¹⁰. We believe that the extended replication slow zone might be due to the lack of a defined symmetry center in a CGG repeat, allowing it to fold into stable structures at multiple points. Notably, for both 40 and 105 CGG repeats, there was no difference in the severity of the replication blockage between different orientations (Fig. 1b). Thus, CGG repeats stall replication fork progression in mammalian cells in a length-dependent, but orientation-independent manner. These results are qualitatively similar to our previous observations in yeast⁹, but the minimal length of the repeat required for replication stalling is higher in mammalian cells, closely matching the threshold length for expansions in humans.

The lack of orientation dependence in replication stalling can be interpreted in two ways: (i) stalling occurs with equal efficiency on both template strands, or (ii) stalling occurs primarily on the lagging strand template upon the formation of equally stable structures by the repetitive CGG and CCG runs. We favor the second interpretation, since formation of an alternative DNA structure requires a DNA segment to become single-stranded, which is intrinsic to the lagging strand template. In this case, the hairpin-like structures are likely to be responsible for fork stalling by the (CGG)_n/(CCG)_n repeat, since they can be formed by both strands of the repeat⁵, in contrast to strand-specific quadruplexes⁶.

Given the similarity between repeat-mediated replication blockage in mammalian and yeast cells, we studied the mechanisms regulating CGG-mediated replication blockage in yeast. We looked at the effect of fork stabilizing proteins on the replication of CGG repeats in a yeast 2-micron plasmid via 2-dimensional gel-electrophoresis⁹. Tof1 is a fork stabilizing protein supporting normal replication¹² and stabilizing replisomes stalled by hydroxyurea treatment¹³. In addition, it promotes fork pausing at protein-mediated replication barriers, such as Fob1-DNA complexes, centromeres, or tRNA genes, by counteracting the Rrm3 helicase¹⁵. Consequently, replication stalling at these sites is absent in Tof1 knockouts¹⁴. In contrast, we found that the strength of the fork stalling at CGG repeats was increased in *Δtof1*, compared to the wild-type strain (Fig. 2). This increase was not due to a general replication slow down in the Tof1 mutant, since the strength of repeat-mediated stalling was normalized to the strength of the Y arc (Supplementary methods and Figure 1). Tof1 mutation similarly affected fork stalling at inverted repeats¹⁰. Thus, Tof1 sustains forks stalled by DNA structures, while its fork pausing, counter-helicase function is limited to protein-mediated barriers.

Yeast Mrc1 forms a complex with Tof1 and Csm3 proteins, which travels with the replication fork¹³. Besides maintaining the integrity of stalled replisomes, Mrc1 mediates replication checkpoint responses¹⁶. We found that the severity of replication stalling at the CGG repeat was increased in the *Δmrc1* compared to the wild type strain (Fig. 2a,b). To distinguish whether this effect is due to the checkpoint or fork-stabilizing function of the protein, we analyzed its separation of function mutant *mrc1^{AQ}*, which cannot be phosphorylated by the checkpoint kinases¹⁶. When we expressed the *mrc1^{AQ}* allele in the *Δmrc1* strain, the severity of repeat-mediated fork stalling was restored to the wild-type level (Fig. 2a,b). Furthermore, repeat-mediated replication stalling in the *Δtof1Δmrc1* double mutant was similar to that in individual mutants. Thus, fork stabilizing, rather than the checkpoint function of the Mrc1 protein helps replisome progress through CGG repeats. By the same token, replisomes stalled at CGG repeats do not seem to trigger an intra-S checkpoint response.

We propose that chromosomal fragility arises when a replication fork stalled at the expanded repeat escapes the S-phase checkpoint (Fig. 3). This could result in the continuation of the cell cycle before the completion of replication around the repeat^{17,18}. As mitosis proceeds, the under-replicated areas would convert into constrictions and/or double-stranded breaks.

Recent data indicate that breakage and instability of trinucleotide repeats is increased in yeast Tof1 and Mrc1 knockouts^{19,20}. Combined with our current data, these results point to the role of fork stalling in repeat instability. Similarly to our observations, Mrc1 prevented repeat contractions independently of its checkpoint function. The checkpoint function, however, specifically prevented repeat expansions. Thus, replication fork stalling may trigger repeat contractions, while expansions may additionally involve a DNA damage checkpoint-inducing event.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Replication fork stalling at CGG repeats in mammalian cells

(a) Fork progression through CGG repeats in *COS-1* fibroblasts. The location of the repeat within the vector is shown above the corresponding gels. The repeat constructs are designated according to the lagging strand template sequence. Bold numbers - the number of repetitive units. Solid arrows - replication stall sites. b. Quantitative analysis of data in (a). Replication stalling was quantified as the ratio between the maximum radioactive count within the bulge normalized to the adjacent arc; error bars show standard deviations.

Fig. 2. Genetic control of replication fork pausing at CGG repeats

a. Replication fork progression through (CGG)₄₀ in the wild type and mutant *S. cerevisiae* strains. Arrows show replication stall sites. **b.** Quantitative analysis of data in (a).

Fig. 3.
Model of chromosomal fragility at expanded CGG repeats.