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DNA Polymerases Eta and Kappa Exchange with the Polymerase Delta Holoenzyme to Complete Common Fragile Site Synthesis

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Abstract

Common fragile sites (CFSs) are inherently unstable genomic loci that are recurrently altered in human tumor cells. Despite their instability, CFS are ubiquitous throughout the human genome and associated with large tumor suppressor genes or oncogenes. CFSs are enriched with repetitive DNA sequences, one feature postulated to explain why these loci are inherently difficult to replicate, and sensitive to replication stress. We have shown that specialized DNA polymerases (Pols) η and κ replicate CFS-derived sequences more efficiently than the replicative Pol δ. However, we lacked an understanding of how these enzymes cooperate to ensure efficient CFS replication. Here, we designed a model of lagging strand replication with RFC loaded PCNA that allows for maximal activity of the four-subunit human Pol δ holoenzyme, Pol η, and Pol κ in polymerase mixing assays. We discovered that Pol η and κ are both able to exchange with Pol δ stalled at repetitive CFS sequences, enhancing Normalized Replication Efficiency. We used this model to test the impact of PCNA mono-ubiquitination on polymerase exchange, and found no change in polymerase cooperativity in CFS replication compared with unmodified PCNA. Finally, we modeled replication stress *in vitro* using aphidicolin and found that Pol δ holoenzyme synthesis was significantly inhibited in a dose-dependent manner, preventing any replication past the CFS. Importantly, Pol η and κ were still proficient in rescuing this stalled Pol δ synthesis, which may explain, in part, the CFS instability phenotype of aphidicolin-treated Pol η and Pol κ-deficient cells. In total, our data support a model wherein Pol δ stalling at CFSs allows for free exchange with a specialized polymerase that is not driven by PCNA.

Keywords

DNA Polymerase η; DNA Polymerase κ; DNA Polymerase δ; PCNA; AT Microsatellite; TLS

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

1. Introduction

Successful genome duplication is an inherent requirement for cellular stability and avoidance of neoplastic transformation. Human cells encode over 15 distinct DNA polymerases with widely varying biochemical properties and accuracies, presumably reflecting the need for enzymatic flexibility during genome duplication and maintenance (1). This flexibility is required during a variety of situations, including by-pass of DNA lesions via the translesion synthesis pathway (TLS), and the replication of repetitive DNA sequences, especially at fragile sites (2). Common fragile sites (CFSs) are mega-base scale regions of the genome prone to instability emanating from numerous factors which generate replication stress (3). These include (but are not limited to) dormant origin deficits, collisions between the replication and transcription machinery, chromatin organization, and sequence content (4–6). CFS are AT rich (>70%) and are enriched for repetitive DNA sequences including [A/T] and [AT/TA] microsatellites (7). This enrichment has led to one predominant model of CFS instability wherein, repetitive DNA sequences cause replication fork stalling and collapse, generating DNA strand breaks (8). While DNA sequences from certain CFSs have been shown to adopt non-B DNA secondary structures (9, 10), this has not been shown for sequences from FRA16D and FRA3B, the two most frequently altered CFSs. However, all of the above factors have the potential to impede DNA polymerases and contribute to the high rate of spontaneous deletions, amplifications, and translocations present at CFSs (11–13).

Despite having numerous DNA polymerases (Pols), the human genome is duplicated primarily by three: Pols α-primase, δ, and ε, of the B-family. These polymerases synthesize DNA with high fidelity and efficiency with the assistance of mismatch repair (1). However, certain DNA lesions and repetitive DNA sequences dramatically reduce both of these parameters leading to the requirement of specialized polymerases (2, 14). Pols η and κ of the Y-family have been extensively studied for their roles in lesion by-pass during TLS. Loss of Pol η is causative for the cancer predisposition syndrome Xeroderma Pigmentosum Variant (XPV), due to Pol η's unique capacity to accurately and efficiently bypass cyclobutane pyrimidine dimers, the major UV lesion (i.e. T-T dimers) (15). Pol κ has been extensively studied in the by-pass of bulky, minor-groove lesions (i.e. benzo[a]pyrene adducts) and confers resistance to cells treated with such compounds (16, 17).

Although the TLS pathway is well conserved, key mechanistic differences exist between *S. cerevisiae* and mammalian cells regarding the role of the PCNA monoubiquitination at K164 (Ub-PCNA). In *S. cerevisiae,* damage-induced Ub-PCNA is essential for UV lesion bypass and mutagenesis (18, 19). The discovery that Y-family polymerases contain ubiquitin binding domains lead to a model in which TLS polymerase binding directly to the Ub moiety of Ub-PCNA is required for lesion bypass (20, 21). Importantly, because Y-family polymerases also have affinity for PCNA through PIP domains, an alternative function postulated for Ub-PCNA is the disruption of protein associations within the replisome in order to facilitate TLS polymerases gaining access to the primer-template junction (19). Biochemical studies using yeast frpolymerases support this model, showing that both replicative polymerase pausing at a lesion and the presence of Ub-PCNA are required for Pol η to replace the highly processive Pol δ (22).

In contrast, numerous studies have shown that Ub-PCNA in mammalian cell TLS functions differently from the model described above for *S. cerevisiae*. Specifically, while Ub-PCNA is formed in mammalian cells after damage, and is important for efficient TLS, a significant proportion of Pol η-mediated TLS is Ub-PCNA independent (23). Indeed, Pol η interactions with PCNA via its PIP domain, but not its interaction with ubiquitin via the UBZ domain, are required for TLS in human cells (24). Additionally, experiments in human cell extracts have shown Pol η TLS across a T-T dimer does not require Ub-PCNA or Rad18 (25, 26). Finally, a recent biochemical study has definitively shown that Pol η/δ exchange at a T-T dimer is independent of PCNA mono-ubiquitination (27). This finding, together the low processivity of the human Pol δ holoenzyme (28, 29), led to a new model wherein human polymerase exchange during TLS is not an active process requiring Ub-PCNA, but rather is a passive process mediated by the intrinsic affinities of polymerases for PCNA and the DNA template (28).

Pols η and κ also have important functions outside of lesion bypass including microsatellite replication, somatic hypermutation, D-loop extension, and G-quadruplex replication (30–35). Loss of Pol η and κ also increases spontaneous and aphidicolin (Aph)- induced CFS instability, highlighting a need to regulate the activities of numerous DNA polymerases, especially during replication stress, to ensure genome duplication (36, 37). In addition to Pols η and κ, humans have two other Y-family polymerases, Pol ι and Rev1, as well as the specialized B-family polymerase, Pol ζ (Reviewed in (2)). While much less is known about these polymerases outside of TLS, Pol ζ has been shown to promote CFS stability (38).

While replication of CFS regions is known to be inherently problematic, a major outstanding question is how various DNA polymerases are orchestrated to ensure their complete replication during normal and stressed conditions. In this study, we designed a model of lagging strand replication to investigate if Pol η and κ can exchange with a stalled Pol δ/PCNA holoenzyme (Pol δHE) at repetitive CFS sequences. Using this model, we determined that Pols η and κ are proficient in rescuing stalled Pol δHE synthesis at both mononucleotide and inverted repeat (IR) sequences, and this rescue was independent of PCNA, and PCNA mono-ubiquitination. We also discovered that this rescue can take place in the presence of Aph, despite significant inhibition of Pol δHE. Our results collectively demonstrate that a stalled replicative polymerase can exchange with a specialized polymerase at repetitive CFS sequences and in the presence of Aph. These findings suggest that the CFS instability phenotype of Pol η and κ-deficient cells may be caused by loss of both efficient repetitive DNA sequence replication and replication stress-resistant synthesis.

2. Materials and Methods

2.1 Replication Proteins, DNA Substrates, and Chemicals

Human Polδ4, PCNA, and K164 mono-ubiquitinated PCNA were purified as previously described (39, 40). We also confirmed the integrity and homogeneity of the PCNA preparations used in this study by immunoblotting (Supplemental Fig 1). Yeast RFC was a generous gift from Linda Bloom (University of Florida). Human Polymerase η and κ were purchased from Enzymax (Lexington, Kentucky). ssDNA templates containing CFS

sequences were generated using a phagemid system and R408 as previously described (41). Aphidicolin was purchased from Sigma, and dissolved in ethanol.

2.2 S1 Nuclease Sensitivity Assay

Plasmid DNA was incubated with or without 1 unit of S1 Nuclease (Life Technologies) for 1, 3, or 5 minutes at 37 °C. Reactions were stopped by adding EDTA and icing, and analyzed by electrophoresis through 0.8% tris-acetate EDTA gels. A plasmid (pJY9) containing a $[TTTC/AAAG]_9$ microsatellite that forms H-DNA was used as a positive control (42). Bands were quanitified with ImageJ (NIH). Nicked and linear intensities were normalized to the supercoiled DNA in each lane.

2.3 PCNA Pre-loading

To accommodate the biochemical requirements of 4 different enzymes, we designed a PCNA pre-loading model (Fig 2). For the pre-loading reactions, 400 fmol of ssDNA hybridized with ^{32}P end-labeled primer was incubated with 400 fmol PCNA and 1700 fmol RFC in buffer containing 20 mM Tris pH 7.5, 8mM $MgCl_2$, 5 mM DTT, 40 ug/mL BSA, 150 mM KCl, 5% glycerol, and 0.5 mM ATP for 5 minutes at 37 °C. The pre-loading reaction was used to reconcile the fact that yeast RFC activity and stability requires high salt (43), while human DNA polymerase perform best at low salt (Figure S1B) (44, 45). For minus (−) Loaded PCNA reactions, all of the above steps were carried out, except RFC was omitted from the reaction.

2.4 Dual Polymerase Reactions and Quantification

An aliquot of preloaded PCNA/DNA substrate (75–100 fmol) was diluted into polymerase reaction buffer containing 25 mM KPO_4 pH 7.6, 5 mM $MgCl_2$, 2.5 mM DTT, 200 ug/ml non-acetylated BSA, and 250 μM dNTPs at 37 °C. Reactions were initiated with 600 fmol Pol δ4. Aliquots of the reaction were removed (T_1) before adding either 500 fmol Pol η, or 250–500 fmol Pol κ, diluted in reaction buffer. For the aphidicolin study, Aph was added with the second polymerase. Reactions were stopped after an additional ten minutes (15 minutes total). Reaction products were separated on denaturing polyacrylamide gels and quantitated using ImageQuant software. %Transit was quantified as [number of molecules in the region 3′ of the CFS] ÷ [total number of molecules in the CFS and the region 3′ of the CFS] x100% (41). To normalize the dual polymerase reactions, the T_1 %Transit (Pol δ alone) was subtracted from the T_2 %Transit, yielding the Normalized Replication Efficiency. For each template, at least 3 independent RFC loading and dual polymerase reactions were performed.

2.5 Stimulation of Pol η

For analysis of Pol η stimulation, reactions were performed by loading PCNA or Ub-PCNA (400 fmol) for three minutes with RFC (1700 fmol) in the above pre-loading buffer plus 250 μM dNTPs. Reactions were initiated by adding Pol (1000 fmol) and stopped with an equal volume of stop dye after 5 and 15 minutes.

2.6 In vitro Genetic Assay

PCNA preloading was carried out as above using 1 pmol of ssDNA, 1 pmol of PCNA, and 4 pmol RFC to generate sufficient DNA product. Reactions were initiated by adding 6 pmol Pol δ4. 5 pmol Pol η was added after 10 minutes and the reaction was stopped by adding EDTA after 30 minutes in total. Reaction products were concentrated before digesting with BamHI. The digested reaction products were ligated back into the pGEM and used to transform DH5αIQ cells. Individual clones were picked and sequenced using CEQ 8000 (Beckman Coulter). Data for Pol δ and η alone reactions were published previously (46)

2.7 Statistical Analysis

Where indicated, data were analyzed by one or two-way ANOVA using GraphPad Prism software with Bonferroni's, Tukey's, or Sidak's multiple comparison post-hoc test. All experiments performed have at minimum three replicates. * = $p<0.05$; ** = $p<0.01$; ***= $p<0.001$; **** = $p<0.0001$.

3. Results

3.1 Repetitive CFS Sequences Display S1 Nuclease Sensitivity

To test if repetitive sequences within CFSs can adopt a non-B DNA structure, we treated plasmids containing inserts of ~150bp from FRA16D or FRA3B (Table 1) with S1 nuclease. Because this enzyme cuts ssDNA preferentially, the production of linear DNA resulting from treatment reflects DNA that has adopted a non-B DNA secondary structure (47). We used plasmids containing a $[TTTC/AAAG]_9$ repeat, which forms H-DNA (triplex) as a positive control in this assay (42). The production of nicked DNA increased with the time of S1 nuclease treatment, and at all timepoints following S1 treatment, linear DNA was detectable (Fig 1A). These results are consistent with H-DNA formation creating an obligate single DNA strand that can be cleaved by S1 nuclease. Our control CFS sequence is derived from FRA16D and is AT rich, but contains no repeats and is not expected to adopted non-B DNA structures (CFS Control). As shown in Figure 1, plasmids with this sequence had some background conversion of super-coiled to nicked DNA with S1 treatment, but no linear DNA was produced. The parental pGEM vector with no insert produced similar results (Data not shown). The AT1 insert sequence contains a polypurine-polypyrimidine $[A/T]_{28}$ repeat that could adopt an H-DNA conformation. Treatment of AT1 plasmids with S1 nuclease caused a clear increase in linear DNA (Fig. 1A, C). Plasmids containing the IR1 and AT3 CFS-derived sequences, which also encode [A/T] repeat sequences, also had detectable levels of linear DNA following S1 treatment (Fig 1C). These results confirm that supercoiled plasmids encoding repeat sequences from FRA16D and FRA3B can adopt a non-B DNA structure in solution, without the need for unwinding.

3.2 PCNA Pre-loading Model

To study polymerase exchange at CFS sequences *in vitro*, we needed to design a biochemical system which allows for optimal activity of multiple enzymes. It has been demonstrated that RFC performs best in high-salt conditions *in vitro* (43), while human

DNA polymerases δ (44), κ (45), and η (Supplemental Fig 1), perform best in low salt. To circumvent this difference, we developed a PCNA preloading model wherein RFC loads PCNA onto primed, ssDNA substrates in high-salt buffer. This loaded substrate is then diluted into a buffer which is optimal for DNA synthesis by all three polymerases (Fig 2A). Reactions are initiated by adding Pol δ4 forming a holoenzyme with PCNA/RFC (Pol δHE). Using our control CFS substrate, we verified equal activity conditions for all three polymerases using the pre-loading model (Fig 2B). With several experiments, we confirmed RFC activity via Pol δ stimulation on different templates (See Fig 2E and Fig 4C).

3.3 Pols η and κ Exchange with Stalled Pol δHE at Inhibitory CFS Sequences

We first tested polymerase exchange on the AT1 template from FRA16D, because we have previously shown this sequence to be highly inhibitory to Pol δ4 (41, 48). For these dual polymerase experiments, all reactions are initiated by Pol δ4 and allowed to progress for 5 minutes. An aliquot is then removed (T_1 Timepoint) and then a second polymerase is added, and reactions are stopped after an additional 10 minutes (T_2 Timepoint). As a control, we initiated synthesis by Pol δ4 and then added additional Pol δ4 after T_1 (δ+δ). Even under these conditions, we observed significant pausing within the mono-nucleotide $[A]_{28}$ and very little product extended beyond the repeat (Fig 2C). In contrast, reactions which received equal activities of Pol η (δ+η) or κ (δ+κ) following T_1 added displayed significantly improved Normalized Replication Efficiency (NRE) over the Pol δ+δ reactions (Fig 2C, D). These results demonstrate a stalled replicative polymerase can exchange with a specialized polymerase at repetitive CFS sequences. As additional controls, we verified that RFC loaded PCNA stimulated Pol δ activity in comparison to reactions without RFC (Fig 2E), and that there was no difference in total primer extension between Pol δ+η, Pol δ+κ, and Pol δ+δ T_1 or T_2 reactions (Data not shown).

To confirm an exchange of catalytic activity under these conditions, we analyzed the errors generated during dual Pol δ+η reactions on a different template (IR1). Pol δHE is also inhibited by this sequence (see Fig 4), and we showed previously that the error frequency for *in vitro* Pol η synthesis on the IR1 template is 2.2×10^{-3} (46). We observed a fairly even distribution of errors in the Pol η mono-polymerase reaction between the repetitive T_{19} and IR_{36} motifs (41%) and non-repetitive, B-DNA (59%) (Fig 3A), and many T to C transition errors that are consistent with Pol η's error signature (30). This distribution is inline with the proportions of the template that were repetitive (37%) and B-DNA (63%). We measured an error frequency of 1.7×10^{-3} in the dual Pol δ+η reaction, a frequency that is similar to Pol η alone, and 20-fold greater than Pol δ alone (Fig 3A, B). Importantly, the distribution of errors from the Pol δ+η dual polymerase reaction was significantly biased towards errors in the repetitive DNA (75%) over non-repetitive DNA (25%) ($p=0.017$, Fischer's Exact Test), with a large number of T to C transitions within the repeats, confirming an exchange of catalytic activity. Consistent with the above biochemical data, our genetic data show that specialized polymerases cooperate with Pol δHE by replicating inhibitory DNA, thereby facilitating efficient replication.

3.4 Polymerase Exchange Does Not Occur When Pol δHE Is Not Sufficiently Inhibited

As indicated above, polymerase exchange was also tested on FRA16D IR1 (Fig 4A). Using this template, we observed that Pol δHE paused strongly at the base of the IR sequence at T_1. Addition of either Pol η or Pol κ to these reactions resulted in efficient extension of these stalled products into the region past the CFS. Pol δ+δ reactions however, displayed impaired progression into the 3′ Region at T_2, and still had product stalled at the IR, leading to a significant reduction in NRE (Fig 4B).

Previous work from our lab has shown the AT3 sequence from FRA3B to be inhibitory to Pol δ at early timepoints (2 and 5 minutes), but after 15 minutes Pol δ can replicate past the $[AT]_{25}$ repeat (46, 48). We used this template as a control with the PCNA preloading model, as a sequence which should not require exchange with Pol δHE for replication. While Pol δHE paused strongly at the $[AT]_{25}$ at T_1 (Fig 4D), Pol δHE was able to replicate through this repeat by T_2 in Pol δ+δ control reactions. Reactions with added Pol η or κ were also competent for AT3 replication, and although they generated longer reaction products, had NREs similar to Pol δ+δ control reactions (Fig 4E). These data suggest that although Pols η and κ are more efficient at replicating certain CFS motifs, they are ancillary when Pol δ4HE is not sufficiently inhibited by the template sequence.

3.5 Neither PCNA, nor Ubiquitinated PCNA Drive Polymerase Exchange at CFS Sequences

One of the most studied interactors of specialized DNA polymerases is K164 mono-ubiquitinated PCNA (Ub-PCNA) (49). We utilized purified Ub-PCNA, which we have previously characterized (26) in our PCNA preloading model to test if this form of PCNA would alter polymerase exchange on CFS sequences (See Supplemental Fig 2 for immunoblot of preparations used in this study). Interestingly, in dual polymerase reactions using the IR1 template, we found no significant enhancement of NRE comparing unmodified PCNA to ubiquitinated PCNA (Fig 4A, B). Indeed, our statistical analysis revealed that while the identity of polymerases significantly impacted NRE (Two-Way ANOVA, $p=0.0001$), the effect of PCNA form was not significant ($p=0.3402$). Recent work from another group also showed Ub-PCNA was not required for Pol η and Pol δ exchange across a T-T dimer (27). Using the AT3 template, we did again did not observed any change in NRE between the three dual polymerase reactions (Fig 4D, E). Interestingly, use of Ub-PCNA did not inhibit the Pol δ+δ reactions on IR1 or AT3, confirming previous reports that Ub-PCNA does not impact Pol δ activity (28, 39).

Our studies of polymerase exchange on IR1 also revealed that Pol δ+η and Pol δ+κ reactions did not require RFC for polymerase exchange (Fig 4C), implying that loaded PCNA is dispensable for polymerase exchange with a stalled Pol δ. We also performed control mono-polymerase reactions on IR1 +/- Loaded PCNA. Here we found Pol η and Pol κ %Transit and primer extension were not statistically affected by RFC loading of PCNA (Supplemental Fig 3). Similar results were found on AT1 (data not shown). To directly determine if a specialized polymerase could be stimulated by PCNA on a CFS template, and if Ub-PCNA would alter this stimulation, we performed reactions in high salt buffer, which hinders Pol η synthesis, allowing stimulation to be detectable. Using the AT1 template, we found that Pol η alone, or with RFC or PCNA, had poor %Transit through the CFS (Fig 5A).

However, by allowing RFC to load PCNA, we found Pol η had a four to five-fold increase in CFS replication. When we repeated this experiment with Ub-PCNA, we observed no statistically significant difference in %Transit in comparison to loaded unmodified PCNA (Fig 5B). Under no conditions did we observe a change in primer extension compared to the Pol η alone reaction (Fig 5C). In total, our data show that PCNA is required for Pol δ CFS replication, as expected, but does not increase Pol η or κ affinity for the primer/template junction. These findings also show that while PCNA can stimulate a specialized polymerase through CFS sequences, it is not driving exchange with a stalled Pol δ.

3.6 In Vitro CFS Replication Requires Specialized Polymerases in the Presence of Aphidicolin

Aph is the classic inducer of CFS instability in cells (3) and is a specific inhibitor of replicative DNA polymerases (50). Why CFS sequences are specifically affected by a general polymerase inhibitor, however, is not known and no studies have shown how Aph impacts polymerase biochemistry at CFS sequences. Although human Pols κ and η have been suggested to be Aph-resistant, this has not been demonstrated directly. Using our CFS control template, we found that, indeed, while Pol δHE is significantly sensitive to Aph, Pols η and κ are Aph-resistant (Fig 6A).

Given this differential sensitivity, we investigated how Aph would impact CFS replication in our *in vitro* model. We performed our dual polymerase reactions as described in Figure 2, modifying the assay to include the addition of Aph with the second polymerase (following T_1). In the presence of Aph, Pol δ4HE synthesis on IR1 was significantly diminished and unable to extend any products beyond the CFS (Fig 6B). Inclusion of Aph also significantly impeded Pol δ4HE synthesis through the AT3 sequence, but to a more moderate extent (Figure 6C). These results show that Pol δHE replication is differentially susceptible to Aph inhibition, depending on the CFS sequence. In addition, Pol δHE inhibition by Aph is additive with inhibition by repetitive sequences. Importantly, addition of either Pol η or Pol κ together with Aph resulted in the efficient extension of stalled Pol δ4HE reaction products, such that the NRE through CFS sequences was not significantly different with or without Aph (Fig 6B, C). Together, these data suggest that when Pol δ4HE is inhibited by both difficult-to-replicate sequences and Aph, Pols η and κ are indispensable for CFS replication.

4. Discussion

In this report, we demonstrate for the first time that specialized DNA polymerases can exchange with a replicative polymerase stalled at CFS sequences (Figs 2, 3, and 4). To measure this, we developed an *in vitro* model of lagging-strand replication with Pol δ/PCNA holoenzyme and DNA template sequences from two different fragile sites that significantly impede Pol δ synthesis (46, 48). We show that plasmids encoding these sequences have increased sensitivity to S1 nuclease treatment, indicating that the repeats adopt non-B DNA structures which extrude ssDNA (Fig 1). We found that either Pol η or κ were capable of rescuing a Pol δHE that is stalled at CFS-derived mononucleotide or inverted repeat sequences (Figs 2 and 4). Importantly, we confirmed that the Pol δHE had extended > 90% of the initial CFS primer-template substrates before addition of the second polymerase on

AT1 and IR1. Our genetic data (Fig 3) revealed an enrichment of Pol η's T to C error signature within the repeat sequences that are inhibitory to Pol δHE, in dual polymerase reactions. This allows us to conclude that when added, Pol η, and by extension Pol κ, performed synthesis on molecules initially extended, then stalled by Pol δ within the CFS repeats, instead of initiating synthesis on unextended molecules. Finally, we analyzed the AT3 template from FRA3B, which is also S1 nuclease sensitive, and slows but does not stall Pol δ synthesis. In this case, we measured no significant difference in the replication efficiency of this template between any combination of polymerases (Fig 4). From this, we can surmise polymerase exchange is only necessary when Pol δ is sufficiently inhibited, despite Pols η and κ being more efficient at replicating repetitive CFS sequences.

Because the role of Ub-PCNA in polymerase exchange at DNA lesions is a matter of current debate, we repeated our analysis of dual polymerase reactions using purified Ub-PCNA that we have previously characterized (26). On the IR1 and AT3 templates, we found no difference in NRE between reactions utilizing unmodified PCNA and Ub-PCNA (Fig 4). Analyses of mono-polymerase reactions on CFS templates showed that both RFC Loaded PCNA and Ub-PCNA can stimulate Pol η and κ synthesis through CFSs to similar extents, without enhancing total primer extension (Fig 5 and Supplemental Fig 3). An earlier report also showed that human Pol η with a UBZ mutation had no defect in stimulation by Ub-PCNA, suggesting Ub-PCNA is not interacting with Pol η in a manner that is different from unmodified PCNA (51). We also found no inhibition of Pol δ CFS synthesis by Ub-PCNA, in comparison to unmodified PCNA (Fig 4). Moreover, in the dual polymerase reactions with Y-family polymerases, we measured no significant difference in synthesis through the CFS sequence, using templates prepared with or without RFC (Fig 4C), suggesting that PCNA loading onto the ssDNA template is dispensable for exchange. Cumulatively, our data concerning human DNA polymerase exchange at CFSs disagrees with the popular TLS model of Ub-PCNA mediated recruitment/displacement (20, 21). Instead, our data support a model wherein dissociation of Pol δ at inhibitory CFS sequences (as we have previously shown in (48)) allows for free exchange with a specialized polymerase, independent of PCNA, and PCNA mono-ubiquitination. Our results are completely consistent with recent biochemical work showing human Pol η TLS across a T-T dimer was also independent of PCNA ubiquitination and is instead driven by Pol η's intrinsic affinity for a primer/template junction (27). Our data are also consistent with work in mammalian cells showing Ub-PCNA independent TLS (23), and the observation that the four-subunit human Pol δ is distributive, while the yeast three-subunit Pol δ is processive (29, 52)

Our biochemical results provide a mechanism to explain the elevated CFS breakage observed in Aph treated, Pol η and Pol κ deficient cells (36, 37, 46). CFS are hot-spots for replication stress induced instability, and Aph is the classic inducer of CFS breakage and replication stress (3). We show here, for the first time, that human Pols η and κ are Aph resistant, while the Pol δHE is Aph sensitive (as expected) (Fig 6A). Using the FRA16D IR1 template, where Pol δHE required Pol η or κ rescue, we found addition of Aph to Pol δ+δ reactions significantly impaired synthesis, resulting in little-to-no extension beyond the IR (Fig 6B). Interestingly, on AT3 where Pol δ+δ reactions perform equally to Pol δ+η and Pol δ+κ reactions, we found that addition of Aph reduced Pol δ+δ NRE in a dose-dependent manner, although not as dramatically as IR1 (Fig 6C). This finding shows

that different repeat sequences have additive inhibition with Aph and can in part, explain why different fragile sites are differentially sensitive to Aph in cells. Importantly, on both templates examined, Pol η and κ were still competent for Pol δHE rescue in the presence of Aph. Together, our results suggest that the CFS instability phenotype of Pol η and κ deficient cells treated with Aph is due to both a loss of efficient repetitive DNA replication, and Aph resistant DNA synthesis.

In contrast to replicative polymerases, Pols η and κ have more open active sites which make little conformational change during catalysis (2). They also possess unique structural features which have been shown to force non-B DNA (due to DNA lesions) into B-form (53, 54). These features could explain each polymerases' ability to efficiently replicate non-B DNA from CFS. While speculative, the differences in active sites between B- and Y-family polymerases may also explain their sensitivity to Aph. Binding of Aph to Pol α was shown to force the active site into an open conformation, distinct from the closed structure of Pol α + dNTP, which is required for catalysis (50). Thus the Aph resistance of Y-family and other specialized polymerases may relate to the fact that these enzymes do not make large conformational changes during catalysis (55).

In summation, our data support the following model for CFS replication. Replicative polymerase stalling at repetitive, non-B DNA sequences leads to polymerase dissociation within CFS regions. This event allows for specialized Pols η or κ to bind the stalled primer/template junction and efficiently replicate through the inhibitory structures. Our data suggest this binding is independent of PCNA or Ub-PCNA. Importantly, several groups have found that PCNA is unloaded from stalled replication forks during stress (56–58). Based on our findings, we propose that removal of PCNA from a stalled fork does not prevent Pol η or κ from resuming stalled synthesis, and may in fact improve access of the specialized polymerases to the primer/template junction.

This model of transient Polδ HE dissociation and passive exchange with a specialized polymerase would be magnified during conditions of replication stress, wherein replicative polymerases will stall more frequently within a single CFS and at additional CFSs (3). While repetitive DNA is a common feature of CFS (3, 7), fragile site instability is cell-type specific (4, 59), likely due to differences in transcription, replication timing, origin usage, and epigenetic programming. Depending on the cell type, a CFS region that utilizes a single origin/replication fork during normal conditions may lack a compensatory fork during replication stress conditions (59, 60). When this is the case, fork restart mechanisms that engage specialized DNA polymerases such as Pols η, κ, and ζ would become indispensable for maintaining CFS stability (37, 38, 46).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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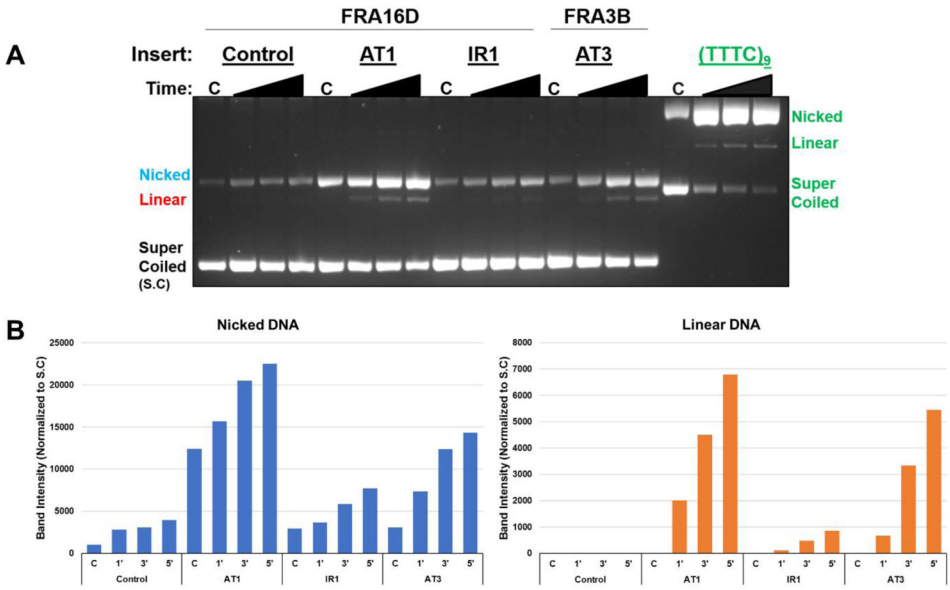

Fig. 1. Plasmids Containing Repetitive Sequences From FRA16D and FRA3B Adopt Non-B DNA Structures

(A) Representative agarose gel of CFS plasmids treated with S1 nuclease for 1–5 minutes. C = plasmid incubated in reaction buffer at 37 °C for 5 minutes without S1 nuclease. See Table 1 for description of CFS insert sequences. Plasmid pJY9 encodes a $[TTTC/AAAG]_9$ microsatellite with H-DNA potential (29) serves as a positive control. (B) The nicked and linear forms for each plasmid at each timepoint were quantified relative to the supercoiled band.

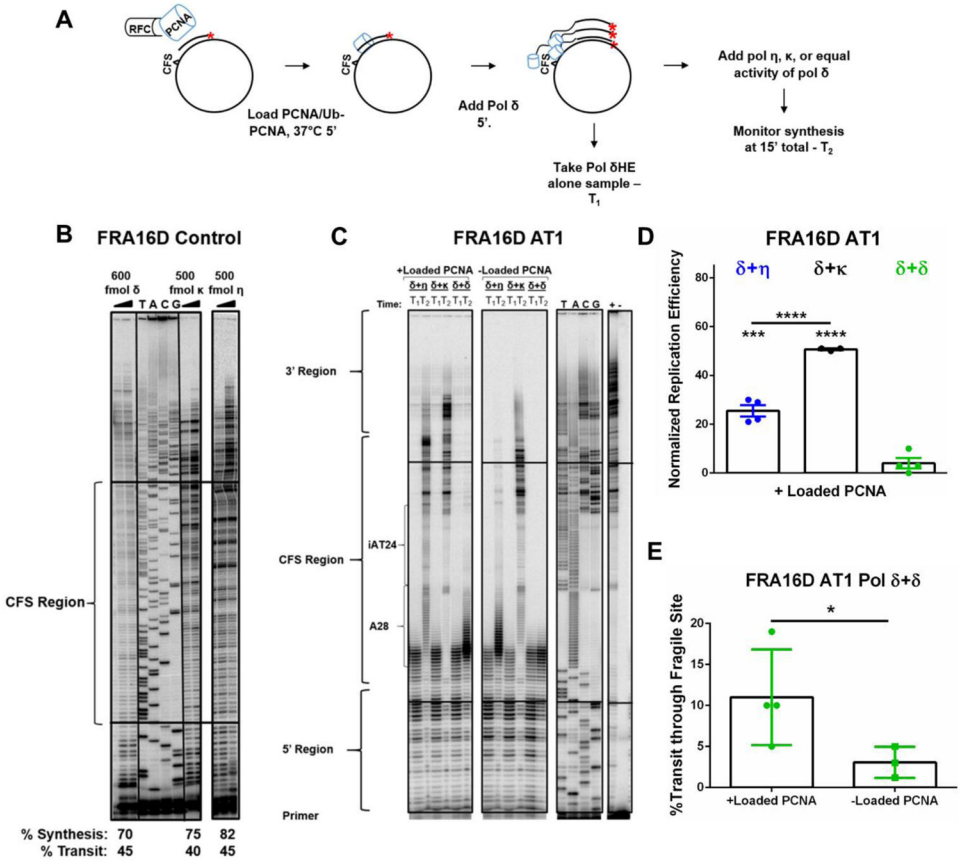

Fig. 2. Pols η and κ Rescue of Stalled Pol δHE Synthesis within CFS sequences

(A) Schematic of PCNA pre-loading and dual polymerase experiments (see text Materials and Methods for details). (B) Mono-polymerase reactions were conducted on FRA16D Control template to establish equal activity conditions. (C) Representative gel of dual polymerase reaction products on FRA16D AT1 with (+Loaded PCNA) or without (-Loaded PCNA) RFC in the preloading reaction. "+" lane is a hybridization control reaction using Klenow Fragment; "–" lane is a negative control lane with no polymerase added. (D) Quantification of Normalized Replication Efficiency (NRE) for N≥3 independent preloading and polymerase reactions. The % transit is quantified as ([products in 3′ region] ÷ [total products CFS + 3′ regions]) x100%. NRE=%Transit (T_2) minus % Transit (T_1), and directly compares the dual polymerase reaction efficiencies by accounting for variability during the pre-loading and T_1 reactions. Individual data points correspond to independent experiments. (E) %Transit quantification for the Pol δ+δ reactions with of without Loaded PCNA. Data are the mean ± SEM, and were analyzed by ANOVA (D) or one-tailed T-Test (E). Significance relative to δ+δ control indicated by asterisks: * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001.

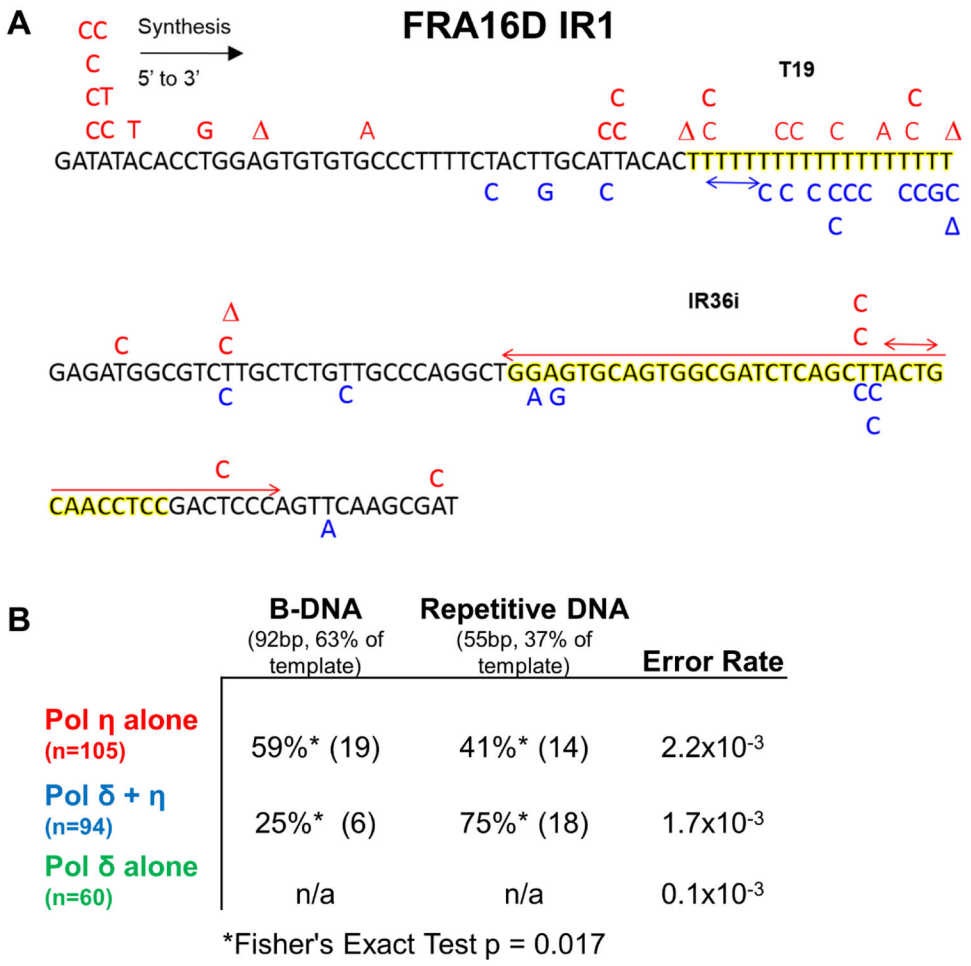

Fig. 3. Pol η Errors are Enriched within CFS-derived Repeat Sequences

(A) FRA16D IR1 sequence (black) with errors made by Pol η alone (above, Red) or Pol δ+η (below, Blue). Repetitive elements highlighted yellow. (B): Analysis of errors created during the indicated polymerase reactions and distribution on the template sequence.

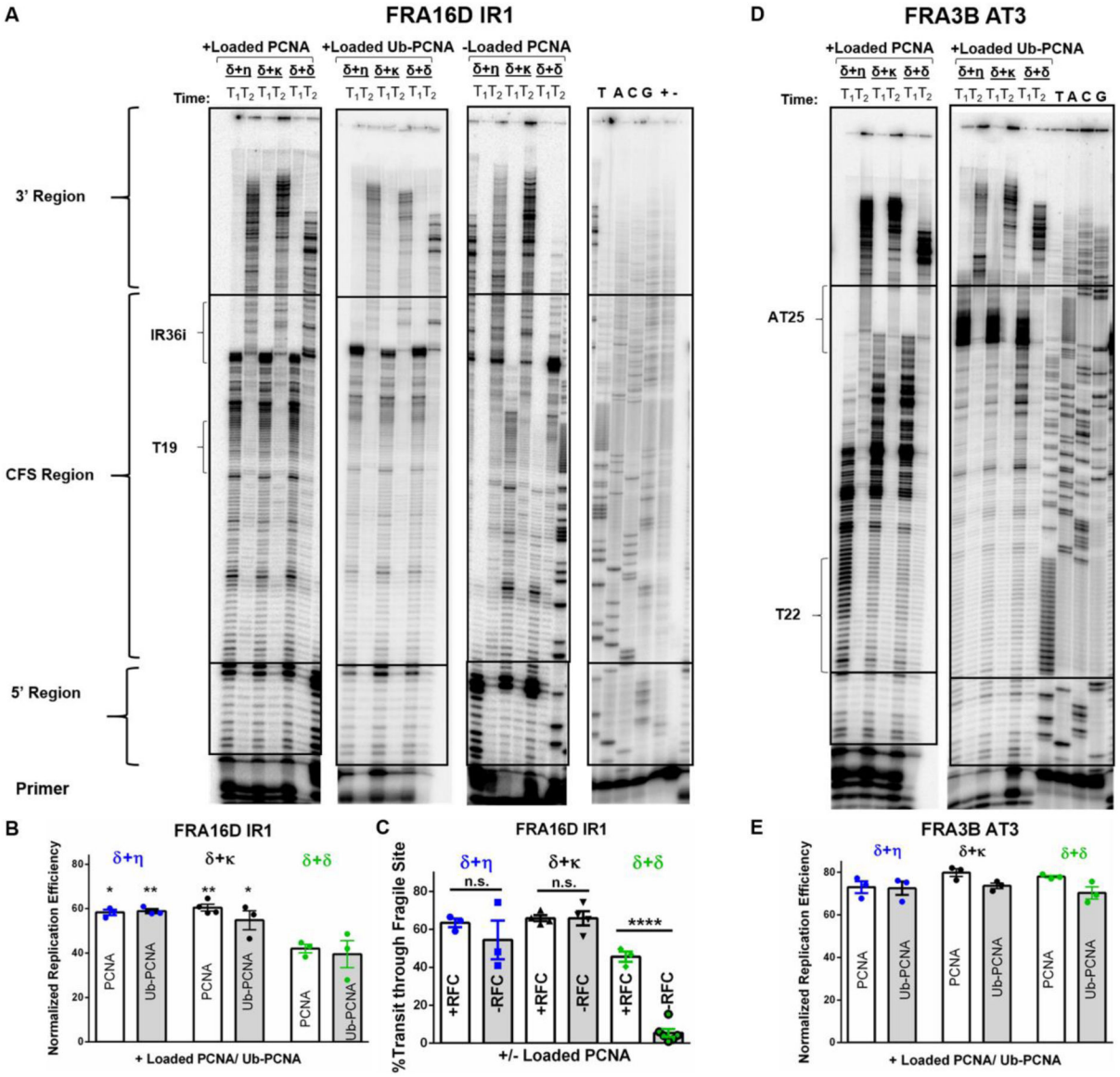

Fig. 4. Rescue of Stalled Pol δHE is Independent of PCNA

(A) Representative gels of dual polymerase reaction products on FRA16D IR1 with Loaded unmodified PCNA, Ub-PCNA, or without Loaded PCNA. (B) Quantification of NRE for the Loaded PCNA and Ub-PCNA reactions. Asterisk (*) above columns indicate statistical significance relative to Pol δ+δ control reactions. (C) Comparison of %Transit values for each dual polymerase reaction with or without Loaded PCNA on the IR1 template. (D) Representative gel of dual polymerase reaction products on AT3 with Loaded PCNA or Ub-PCNA. (E) Quantification of NRE for the Loaded PCNA and Ub-PCNA reactions. Data represent the mean of N≥3 reactions with individual points plotted. All data were analyzed by one- or two-way ANOVA with Tukey's post-hoc.

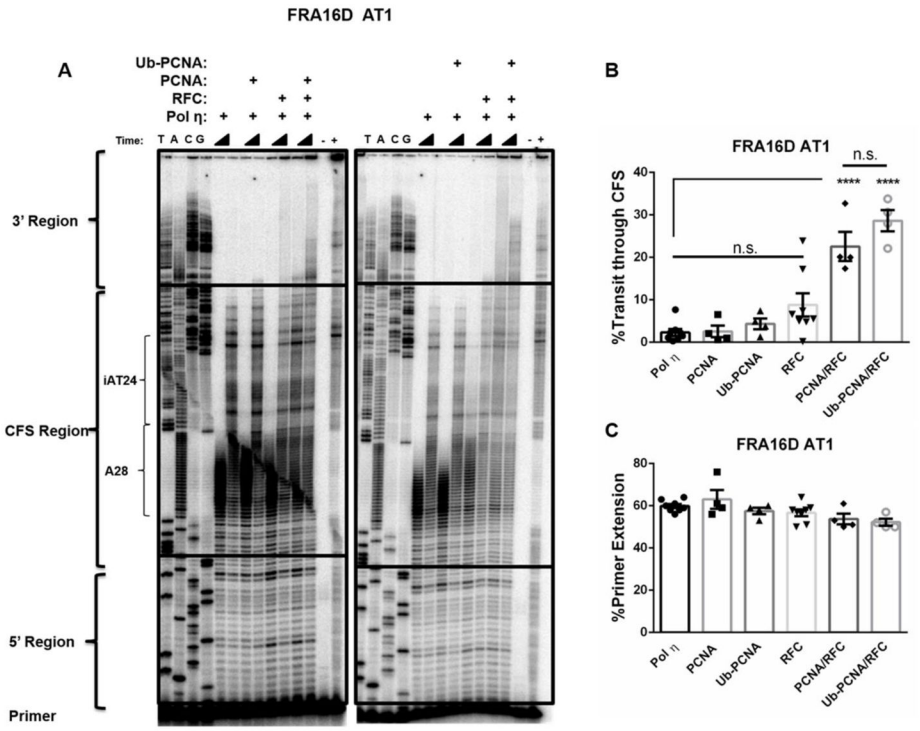

Fig. 5. Pol η CFS Synthesis Can be Stimulated by PCNA

(A) Representative gels of Pol η reactions performed in high salt buffer to detect stimulation using the AT1 template. All reactions contained Pol η and additional protein(s) as indicated. (B, C) Quantitation of %Transit and Primer Extension at the 15 minute timepoint. Data are the mean of N≥3 ± SEM, with individual reaction points shown, and were analyzed by one-way ANOVA.

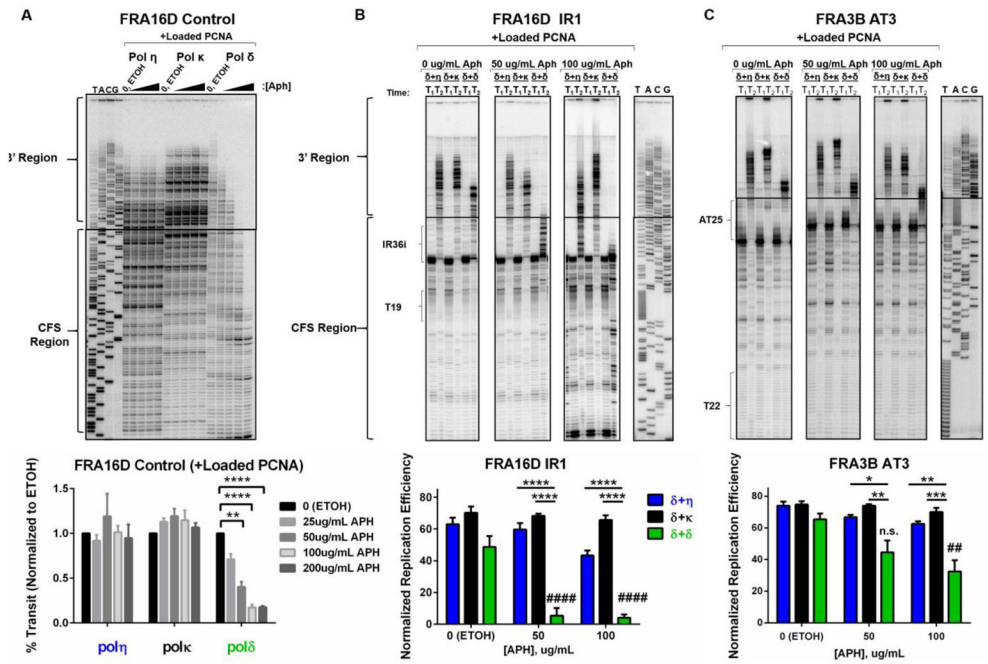

Fig. 6. Pols η and κ are Indispensable for CFS Replication in the Presence of Aph

(A) Top: Representative gel of synthesis products for each polymerase with increasing amounts of Aph (control template). PCNA was preloaded as in Fig 5A. Bottom: Quantitation of %Transit relative to EtOH solvent (N=3). (B, C) Top: Representative gels of dual polymerase reactions with Aph added with the second polymerase (after T1). Bottom: NRE (mean ± SEM) for N>3 independent experiments. # = statistical significance relative to EtOH; *, statistical significance relative to δ+δ.

Table 1

Description of CFS-derived insert sequences

Template[a]	CFS[b]	Repeat Elements	%A/T
Control	FRA16D 199256-199375	None	76%
AT1	FRA16D 191565-191712	$[A/T]_{28}$, $[AT/TA]_{24i}$ Flextab peak 5	74%
IR 1	FRA16D 191713-191860	$[A/T]_{19}$, IR_{36}	53%
AT 3	FRA3B 837821-837929; 837963-838030	$[A/T]_{22}$, $[AT/TA]_{25}$	80%

[a] See ref. 35 for complete sequences.

[b] Genomic coordinates of insert. FRA16D GenBank Accession Number: **AF217490**. FRA3B Genbank Accession Number: **183583557**.