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Sequence-specific stalling of DNA polymerase γ and the effects of mutations causing progressive ophthalmoplegia†

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A large number of mutations in the gene encoding the catalytic subunit of mitochondrial DNA polymerase γ (POLγA) cause human disease. The Y955C mutation is common and leads to a dominant disease with progressive external ophthalmoplegia and other symptoms. The biochemical effect of the Y955C mutation has been extensively studied and it has been reported to lower enzyme processivity due to decreased capacity to utilize dNTPs. However, it is unclear why this biochemical defect leads to a dominant disease. Consistent with previous reports, we show here that the POLγA:Y955C enzyme only synthesizes short DNA products at dNTP concentrations that are sufficient for proper function of wild-type POLγA. In addition, we find that this phenotype is overcome by increasing the dNTP concentration, e.g. dATP. At low dATP concentrations, the POLγA:Y955C enzyme stalls at dATP insertion sites and instead enters a polymerase/exonuclease idling mode. The POLγA:Y955C enzyme will compete with wild-type POLγA for primer utilization, and this will result in a heterogeneous population of short and long DNA replication products. In addition, there is a possibility that POLγA:Y955C is recruited to nicks of mtDNA and there enters an idling mode preventing ligation. Our results provide a novel explanation for the dominant mtDNA replication phenotypes seen in patients harboring the Y955C mutation, including the existence of site-specific stalling. Our data may also explain why mutations that disturb dATP pools can be especially deleterious for mtDNA synthesis.

INTRODUCTION

Nuclear DNA is maintained by a number of specialized DNA polymerases, which function in DNA replication, recombination and repair. In contrast, human mitochondria contain only one DNA polymerase, DNA polymerase γ (POLγ), that consists of one catalytic subunit (POLγA) and two accessory subunits (POLγB) (1). POLγA belongs to the family A DNA polymerases, and the polymerase domain has a 'right-hand' configuration with thumb, palm and fingers subdomains. The polymerase also contains a 3′ to 5′ exonuclease domain that is connected to the polymerase domain by a spacer region (2–4). The exonuclease activity is required for

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proofreading (5). Similar to other DNA polymerases, POLγ most likely alternates between polymerizing and editing modes, and there is a competition between the two active sites for the 3′-primer end of the DNA.

More than 150 disease-causing mutations have been identified in the POLγA gene (http://tools.niehs.nih.gov/polg/). These amino acid substitutions are distributed over the three domains and can lead to depletion and/or accumulation of multiple deletions in mtDNA. Certain POLγA mutations may generate depletion, whereas other POLγA mutations cause deletions in the mtDNA. The biochemical basis for these variations is poorly understood and is not only of academic interest, since specific defects have been linked to different clinical disorders. Good examples are the autosomal dominant and autosomal recessive forms of progressive external ophthalmoplegia (adPEO and arPEO), which are among the most common mitochondrial maintenance disorders (6). PEO is characterized by the accumulation of multiple deleted mtDNA molecules in postmitotic tissues (7,8).

Most of the mutations causing adPEO have been localized to the polymerase domain of POLγA, whereas mutations leading to arPEO are more evenly distributed over the entire enzyme. The most common of all adPEO-causing mutations, Y955C, changes a conserved residue in the finger subdomain of the polymerase domain. Since its discovery in 2001 (9), the biochemical consequences of the Y955C mutation for POLγA function have been characterized in considerable detail (10,11). One striking phenotype of the Y955C POLγA is a 45-fold decrease in binding affinity for the incoming nucleoside triphosphate, which in turn results in a 10- to 100-fold increase in mis-insertion errors (10). The effective intrinsic proofreading activity of the enzyme does however partially compensate for this effect and the Y955C mutation only results in a 2-fold increase in base pair substitutions *in vitro* (10). Another molecular phenotype associated with Y955C is a more than 100-fold decrease in DNA polymerase activity, coupled to a severe decrease in processivity (12). Human POLγA containing the Y955C amino acid substitution has been overexpressed in the heart of transgenic mice, resulting in mtDNA depletion, oxidative stress and cardiomyopathy, supporting its impact on replication (13).

An example of an arPEO-causing mutation is the glycine to serine substitution at position 848, which is situated in the thumb subdomain of POLγA (14). Patients homozygous for G848S have not been identified, but the mutation has been found in trans with other POLγ mutations in patients with different clinical symptoms. Previous reports have demonstrated that this mutation displays a 99% reduction in polymerase activity and a 5-fold decrease in DNA-binding affinity compared with the wild-type protein (15).

In vivo, POLγ cooperates with a number of other enzymes to replicate mtDNA. Among these other enzymes is the TWINKLE protein, which is a hexameric DNA helicase required for DNA synthesis on double-stranded DNA templates (16,17). The reaction is further stimulated by the mitochondrial single-stranded DNA-binding protein, mtSSB, and we have recently demonstrated that the mitochondrial RNA polymerase (POLRMT) plays an essential role as a primase required for origin-specific initiation of lagging-strand DNA synthesis (18,19). The delicate interplay between different replication factors may also influence the outcome of individual mutations in, for example, POLγA.

In this work, we have used the reconstituted mammalian mtDNA replisome *in vitro* and studied functional consequences of the dominant Y955C and recessive G848S PEO-causing mutations (16). In agreement with their *in vivo* effects, we find that the G848S mutation fails to affect replisome activity in the presence of wild-type POLγA, whereas the Y955C mutant POLγA displays a dominant negative effect on replisome function. We have characterized the molecular basis for this interesting difference in molecular detail and demonstrate that the Y955C mutant POLγA is able to compete for the primer template in the presence of the wild-type protein and also shows impairment of the delicate balance between the polymerase and exonuclease modes of the enzyme.

RESULTS

The dominant nature of the Y955C mutation can be reconstituted *in vivo*

To verify the dominant nature of the Y955C mutation *in vivo*, we generated two stable cell lines for inducible expression of either the wild-type POLγA or the POLγA:Y955C mutant variant as described previously (20). We used immunoblotting to confirm overexpression of POLγA after addition of doxycycline to the cell culture medium. The level of POLγA expression was dependent on the doxycycline dose and at 3 ng/ml doxycycline, recombinant proteins were expressed at levels similar to the endogenous POLγA (20) (data not shown). In order to investigate the dominant effect of POLγA:Y955C, the relative mtDNA copy number in the inducible cell lines was compared by quantitative real-time PCR (QPCR) (Fig. 1A). Already low levels of POLγA:Y955C (1 ng/ml doxycycline) caused about 60% decrease in mtDNA copy number, and the effect was even more pronounced when cells were grown in the presence of higher doxycycline concentrations. Overexpression of wild-type POLγA did not affect the mtDNA levels. These results thus support the dominant effect of POLγA:Y955C seen in patients. We next investigated whether replication stalling caused a decrease in mtDNA copy number. We therefore analyzed mtDNA replication intermediates at different POLγA:Y955C concentrations using 2-dimensional neutral/neutral agarose electrophoresis (20,21). All POLγA:Y955C samples showed a replication intermediates pattern, indicating a slight replication-stalling phenotype, and increasing concentrations of POLγA:Y955C caused a progressive decrease in the 7S-containing D-loop (for details, see Supplementary Material, Fig. S1) (22).

The dominant nature of the Y955C mutation may be reconstituted *in vitro*

We next set out to characterize the dominant nature of Y955C *in vitro*, and for comparison, we also analyzed the recessive mutation G848S. Human POLγA and the mutant derivatives POLγA:Y955C and POLγA:G848S were therefore expressed in insect cells and purified as described previously (16). The mutant proteins remained soluble during purification and

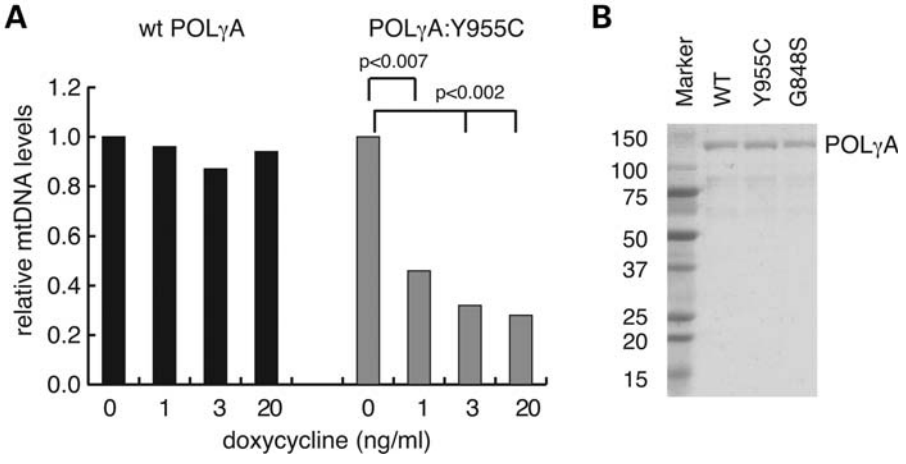

Figure 1. *In vivo* characterization of PEO-causing mutations and purification of recombinant POLγA. (**A**) Induced expression of human POLγA:Y955C results in mtDNA depletion in 293 Flp-In TRex cells. mtDNA levels of wild-type POLγA or POLγA:Y955C; cells were induced with 0, 1, 3 or 20 ng/ml doxycycline for 2–3 days, quantified by duplex Taqman QPCR (20) and normalized to non-induced cells of the same cell line. Both non-induced wild-type and POLγA:Y955C showed mtDNA copy numbers similar to each other and to the parental cell line. Paired *t*-test showed significant copy-number depletion for induced Y955C cells compared with non-induced cells (*P*-values shown) as well as compared with the induced wild-type cells. Four independently isolated samples were measured in triplicate. (**B**) Purified recombinant wild-type and mutant versions of POLγA (0.5 μg) were separated by SDS–PAGE and revealed with Coomassie brilliant blue staining. Lane 1, size marker; lane 2, wild-type POLγA; lane 3, POLγA:Y955C; lane 4, POLγA:G848S.

displayed roughly the same chromatographic behavior as the wild-type protein. The purity of the proteins is shown in Figure 1B. Others have investigated the DNA-binding and exonuclease activities of POLγA:Y955C and POLγA:G848S (12,15). In agreement with these previous studies, our POLγA:Y955C could bind a primed DNA template as efficient as the wild-type POLγA, whereas POLγA:G848S failed to form stable interactions, even in the presence of POLγB (Supplementary Material, Fig. S2A). In the absence of nucleotides, both POLγA:Y955C and POLγA:G848S displayed wild-type levels of exonuclease activity, in spite of the DNA-binding defect associated with the G848S amino acid substitution (Supplementary Material, Fig. S2B).

Both POLγA:G848S and POLγA:Y955C have low polymerase activities on a single-stranded DNA template (12,15). *In vivo*, however, only POLγA:Y955C generates disease phenotypes in the heterozygous state, whereas the G848S amino acid substitution is well tolerated in the presence of wild-type POLγA (14). In order to understand the recessive and dominant nature of these two mutations, we studied the polymerase activity of POLγA:G848S and POLγA:Y955C in the presence of the wild-type protein. To create a template, we hybridized a 60 nt oligonucleotide to a single-stranded pBluescript II KS + plasmid. POLγ may use the free 3′-end of this template to initiate DNA synthesis on the single-stranded plasmid. We incubated this template with wild-type POLγ and mutant derivatives as indicated in Figure 2. On their own, neither POLγA:G848S (Fig. 2A, lanes 6–10) nor POLγA:Y955C (Fig. 2B, lanes 6–10) could support effective DNA synthesis in this assay. When we mixed the mutant and wild-type POLγA at a 1:1 ratio, similar to the *in vivo* situation in the heterozygous state, the wild-type polymerase activity was nearly unaffected by the presence of POLγA:G848S (compare lanes 1–5 with lanes 11–15 in Fig. 2A), whereas POLγA:Y955C repressed wild-type POLγA-dependent DNA synthesis (Fig. 2B, lanes 11–15). We could thus reconstitute the dominant and the recessive effects of Y955C and G848S *in vitro*, using single-stranded DNA as a template.

The dominant effect of the Y955C mutation may be explained by competition with the wild-type POLγA for access to the primer template (15). We could examine this hypothesis by gradually increasing the molar ratio of the wild-type protein relative to the POLγA:Y955C mutant polymerase. In a competitive situation, we would expect to observe DNA synthesis at higher concentrations of the wild-type protein relative to the Y955C variant. Indeed, in the presence of higher wild-type protein concentrations, we observed DNA synthesis, supporting the idea of competition for the DNA template between the wild-type and POLγA:Y955C mutant protein (Fig. 2C).

Effects of the Y955C mutation in the context of the mitochondrial replisome

To replicate duplex DNA, POLγ requires the helicase activity of TWINKLE, and the reaction is further stimulated by mtSSB. The presence of these other factors stabilizes POLγ interactions at the replication fork and allows for the formation of much longer DNA synthesis products (16). We now addressed the effects of POLγA:G848S and POLγA:Y955C in the context of the mitochondrial replisome (Fig. 3). In contrast to the wild-type polymerase, neither POLγA:G848S nor POLγA:Y955C could effectively support DNA synthesis on the dsDNA template in the presence of TWINKLE and mtSSB, even if we could see the formation of some DNA synthesis products (Fig. 3A and B, lanes 6–10). We next examined whether POLγA:G848S or POLγA:Y955C could affect the DNA synthesis activity of the wild-type POLγA when added at a 1:1 ratio. In agreement with its recessive nature *in vivo*, POLγA:G848S failed to inhibit the wild-type protein (Fig. 3A, lanes 11–15). In contrast, POLγA:Y955C caused a strong decrease in DNA synthesis, but the protein

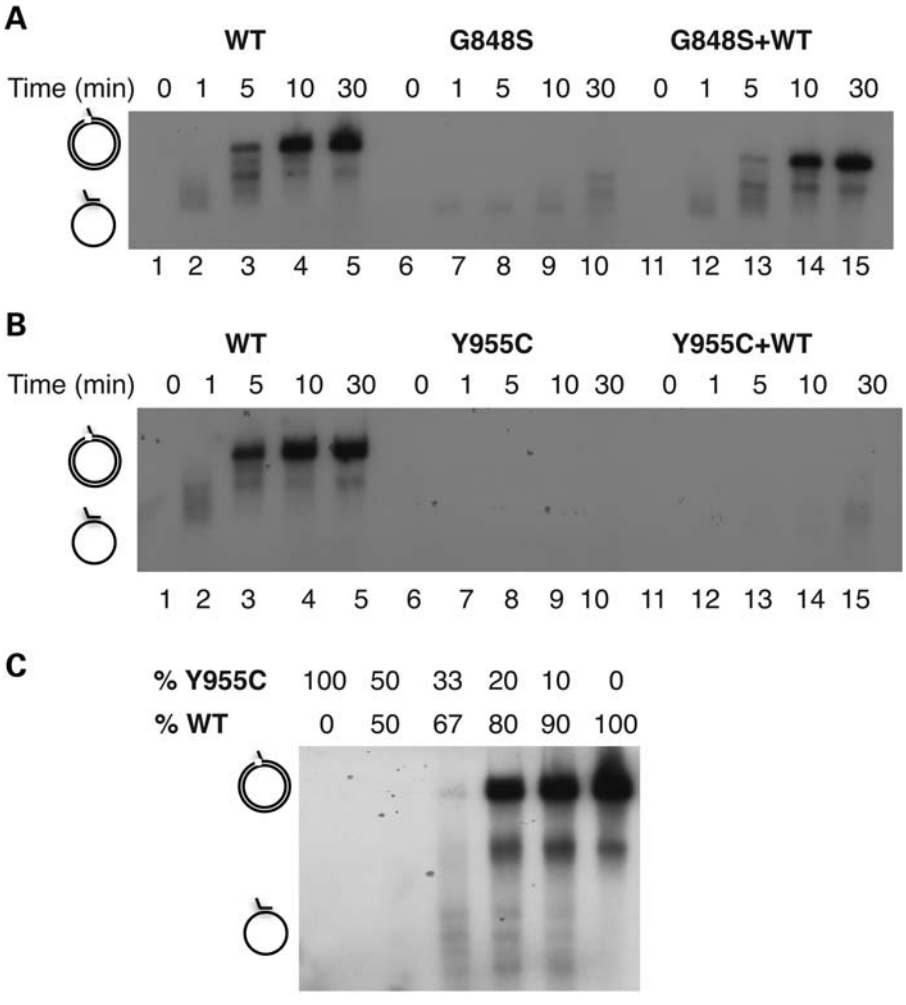

Figure 2. POLγA:Y995C displays a dominant negative effect on the polymerase activity of the wild-type POLγA. DNA synthesis rates were monitored using a primed circular single-stranded DNA template (~3000 bases). (**A**) A comparison between wild-type POLγA and POLγA:G848S. The indicated versions of POLγA (150 fmol) and POLγB (300 fmol) were incubated with the primed template (10 fmol) at 37°C. Samples were taken at the indicated time points and analyzed on a 1% native agarose gel. (**B**) The analysis was performed as in (A), but with wild-type POLγA and POLγA:Y955C. (**C**) An increase in the relative concentration of POLγA:Y955C versus POLγA inhibits DNA synthesis. The measurements were performed as described for (A) and the reactions were incubated for 30 min at 37°C.

did not completely abolish full-length DNA synthesis (Fig. 3B, lanes 11–15). These results thus contrasted to the results obtained with the single-stranded template, where no full-length products were observed after the addition of POLγA:Y955C (Fig. 2B). A close examination suggested the existence of two populations of DNA synthesis products, one consisting of shorter products, similar to that seen with POLγA:Y955C in isolation, and a second group of longer DNA products. The longer products are similar to those seen with wild-type POLγA; however, the amount of these longer replication products was severely decreased compared with the wild-type situation (Fig. 3B, lane 15). The results seemed to indicate that wild-type POLγA and POLγA:Y955C may function independently of each other, i.e. once DNA replication is initiated, the process is processive and not affected by the presence of mutant POLγA:Y955C.

To address this possibility, we initiated DNA synthesis on the double-stranded template using a constant amount of POLγ together with TWINKLE and mtSSB. All reactions were allowed to proceed for 60 min and POLγ:Y955C was added at a 3-fold excess relative the wild-type POLγ at the times indicated in the figure (Fig. 3C). The data revealed that POLγ:Y955C exhibited a dominant negative effect on DNA synthesis if the protein was added at the onset of the reaction (Fig. 3C, lane 7), but if the wild-type replisome had been able to initiate replication, the effect was less profound and we could still observe the formation of full-length DNA products (Fig. 3C, lanes 8 and 9). The most straight-forward explanation for this observation is that POLγ is processive in the replisome context and that POLγ:Y955C added after initiation of DNA synthesis fails to compete with the wild-type protein for access to the primed template.

Y955C requires higher dNTP concentration to synthesize DNA

We next asked whether it was possible to overcome the non-processive nature of POLγA:Y955C. Others have reported

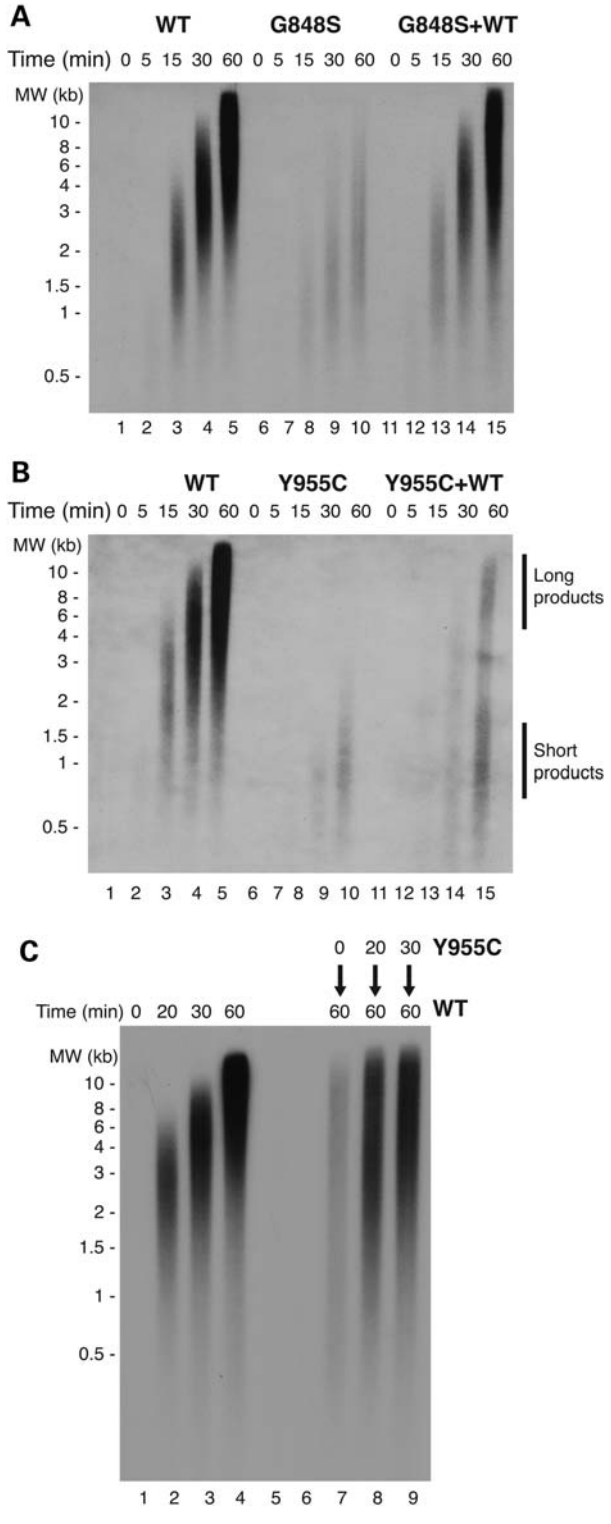

Figure 3. Wild-type POLγA and POLγA:Y955C can function independently of each other. DNA synthesis rate was measured using a mini-circle template. Indicated POLγA versions (final amount 100 fmol), POLγB (150 fmol as a dimer), TWINKLE (100 fmol) and mtSSB (5 pmol) were incubated with the mini-circle template (10 fmol) at 37°C as described in Materials and Methods. Samples were taken at the times indicated, and analyzed on a 0.8% denaturating agarose gel. In (**A**), wild-type POLγA and POLγA:G848S DNA synthesis were analyzed and in (**B**), wild-type POLγA and POLγA:Y955C DNA synthesis were analyzed in isolation and in combination. (**C**) A competition experiment was carried out as described in Material and Methods using the same template as in (A) and (B). TWINKLE (100 fmol), mtSSB (5 pmol) and wild-type POLγ (100 fmol POLγA and 150 fmol as a dimer POLγB) were incubated at 37°C for 60 min. At the times indicated, POLγ:Y955C (300 fmol POLγA and 450 fmol POLγB) was added. Lanes 1–4, control experiments with wild-type POLγ were stopped at times indicated; lane 7, POLγ:Y955C was added to the wild-type reaction after 0 min incubation; lane 8, POLγ:Y955C was added to the wild-type reaction after 20 min incubation; lane 9, POLγ:Y955C was added to the wild-type reaction after 30 min incubation.

that this mutant form exhibits a 350-fold increase in the K_m for dTTP insertion compared with the wild-type polymerase (10). These experimental findings are in nice agreement with a recent structural study which has demonstrated that Tyr955 together with Glu895 and Tyr951 forms a hydrophobic pocket within POLγA that accommodates and stabilizes the incoming dNTP during DNA synthesis (23). In addition, a phenotype, induced by an amino acid change in the yeast mitochondrial DNA polymerase corresponding to Y955C, can be suppressed by increasing the mitochondrial dNTP pool by overexpression of *RNR1*, encoding the ribonucleotide reductase (*RNR1*) (24). These findings suggested that POLγA:Y955C may require higher dNTP concentrations in order to function properly. We therefore repeated the replisome experiment, but in the presence of increasing amounts of dNTPs.

Interestingly, higher dNTP concentrations did not significantly affect wild-type POLγA (Fig. 4A), but caused a dramatic increase in POLγA:Y955C-dependent DNA synthesis (Fig. 4B). At high dNTP concentrations, the products of POLγA:Y955C DNA synthesis were of similar size as those obtained with the wild-type POLγA. We could thus conclude that an increase in dNTP concentrations could rescue the POLγA:Y955C phenotype *in vitro*. We also repeated this experiment, but now with both wild-type POLγA and POLγA:Y955C added at a 1:1 ratio (Fig. 4C). At lower dNTP concentrations, we could observe low levels of full-length DNA synthesis (Fig. 4C, lanes 2 and 3) probably produced by the wild-type POLγA. At intermediate dNTP concentrations, we could observe the low levels of full-length DNA synthesis, as well as a population of shorter DNA products, similar to what has been described above (Fig. 4C, lanes 4 and 5). At the highest dNTP concentrations, the shorter DNA products disappeared and we instead observed a strong increase in full-length DNA synthesis (Fig. 4C, lanes 6 and 7). Hence, the result of this experiment resembles a superimposition of the experiments with either wild-type or POLγA:Y955C in isolation (Fig. 4A and B). The experiments thus again demonstrated that once DNA synthesis had been initiated, wild-type POLγA can function in the presence of POLγA:Y955C. Furthermore, given sufficiently high dNTP concentration, POLγA:Y955C may also be highly processive.

A specific requirement for higher dATP concentrations

We next asked whether the requirement for higher dNTP concentrations was a general phenomenon or specific for certain dNTPs and whether POLγA:Y955C stalled at specific sites

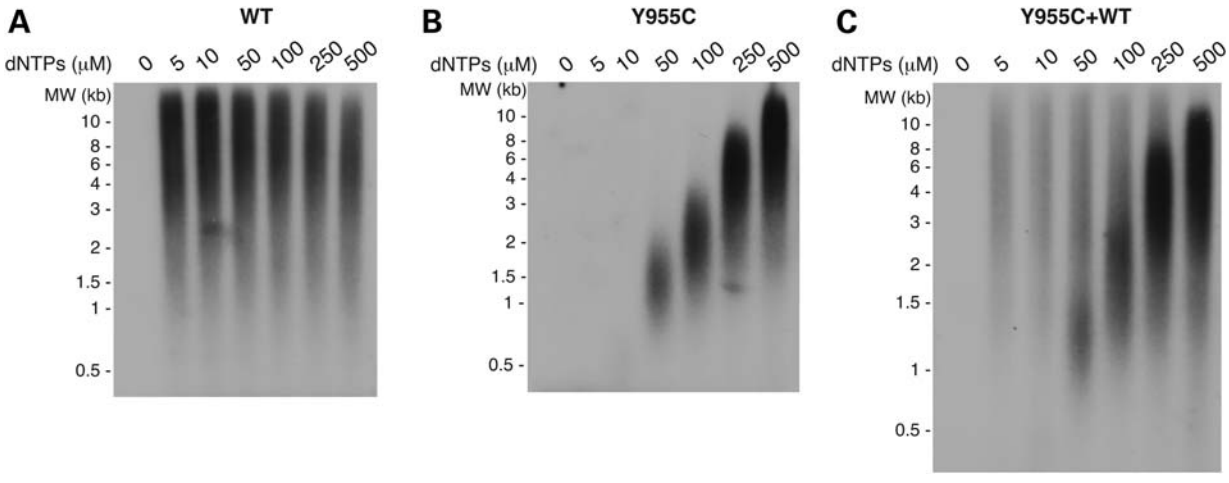

Figure 4. POLγA:Y955C is processive at high dNTP levels. The reactions were carried out as in Figure 3 but in the presence of indicated concentration of dNTPs, and all reactions were incubated for 40 min at 37°C and analyzed as described in Material and Methods. (**A**) Wild-type POLγA alone, (**B**) POLγA:Y955C alone and (**C**) wild-type POLγA and POLγA:Y955C in combination.

(Fig. 5). To monitor this, we used a radioactively labeled primed DNA substrate in the presence of different dNTP concentrations. In the absence of dNTPs, wild-type POLγ exhibits exonuclease activity, but upon addition of dNTPs, the polymerase shifted into the polymerase mode and elongated the DNA primer (Fig. 5A). POLγA:G848S behaved exactly as wild-type POLγA, and we decided not to investigate this mutant in further detail (data not shown). POLγA:Y955C displayed wild-type levels of exonuclease activity in the absence dNTP, and a similar dNTP concentration was required to block the exonuclease activity (Fig. 5A). In contrast to the wild-type protein, POLγA:Y955C required much higher dNTP concentrations to fill out the 15 nt single-stranded region of the template (Fig. 5A, compare lanes 3–6 and 11–14). The wild-type POLγA required about 1 μM of dNTPs, whereas POLγA:Y955C needed a 100-fold higher concentration (about 100 μM) for full-length DNA synthesis. These findings are in agreement with the replisome experiments.

To investigate whether this effect was caused by any specific dNTP, we repeated the experiment in the presence of relatively low dNTP concentration (1 μM) and increased the concentrations of individual dNTPs (200 μM). Our experiments demonstrated that POLγA:Y955C could tolerate low dGTP, dCTP and dTTP concentration, but failed to produce full-length DNA synthesis products in the presence of low dATP concentrations (Fig. 5B). Our data suggested that POLγA:Y955C has a specific defect in dATP incorporation. To address this possibility, we repeated the same experiment, but using the wild-type POLγA. To mimic the situation with POLγA:Y955C, we used lower dNTP concentrations (0.1 μM) to prevent full-length DNA synthesis. At these conditions, wild-type POLγA failed to produce full-length products, but addition of high dATP concentrations allowed for full-length DNA synthesis to occur. Therefore, wild-type POLγA behaved in a manner identical to that observed with POLγA:Y955C; they both required higher dATP concentrations even if the relative dNTP concentrations required are much higher for the POLγA:Y955C enzyme.

We noted an interesting anomaly in DNA synthesis experiments performed in the presence of increasing dNTP concentrations. Even at concentrations that completely blocked exonuclease activities, the POLγA:Y955C enzyme did not switch over to full-length DNA synthesis. Instead, the reactions only proceeded for a couple of nucleotides, after which they were stalled (Fig. 5B). It appeared as if POLγA:Y955C DNA synthesis was terminated in a site-specific manner. We mapped the exact site for the DNA synthesis termination and found that the DNA synthesis reaction was terminated just before incorporation of the first dATP (Fig. 5C). This finding was thus in nice agreement with the observation that POLγA:Y955C is sensitive to low dATP concentrations. To confirm that this result was not template-dependent (since the template used in these experiments contained a poly-dT_{10} stretch), we performed the stalling experiment at low dATP concentrations using a template with a mixed-sequence single-stranded stretch (Supplementary Material, Fig. S2C). Also here, we noted site-specific termination at the first site of dATP incorporation.

The molecular basis for the dominant phenotype of POLγA:Y955C

Our initial experiments on a single-stranded DNA template had supported a dominant effect of POLγA:Y955C, but our later experiments in the context of the replisome suggested that the wild-type and mutant DNA polymerase could function independently of each other, i.e. the wild-type polymerase was processive also in the presence of POLγA:Y955C. To understand this apparent contradiction, we decided to investigate the effects of the two enzymes in order-of-addition experiments. First we compared wild-type POLγA and POLγA:Y955C in a time-course experiment at a dNTP concentration of 1 μM and found that the wild polymerase had completed synthesis already after 1 min (Fig. 6A, lane 2).

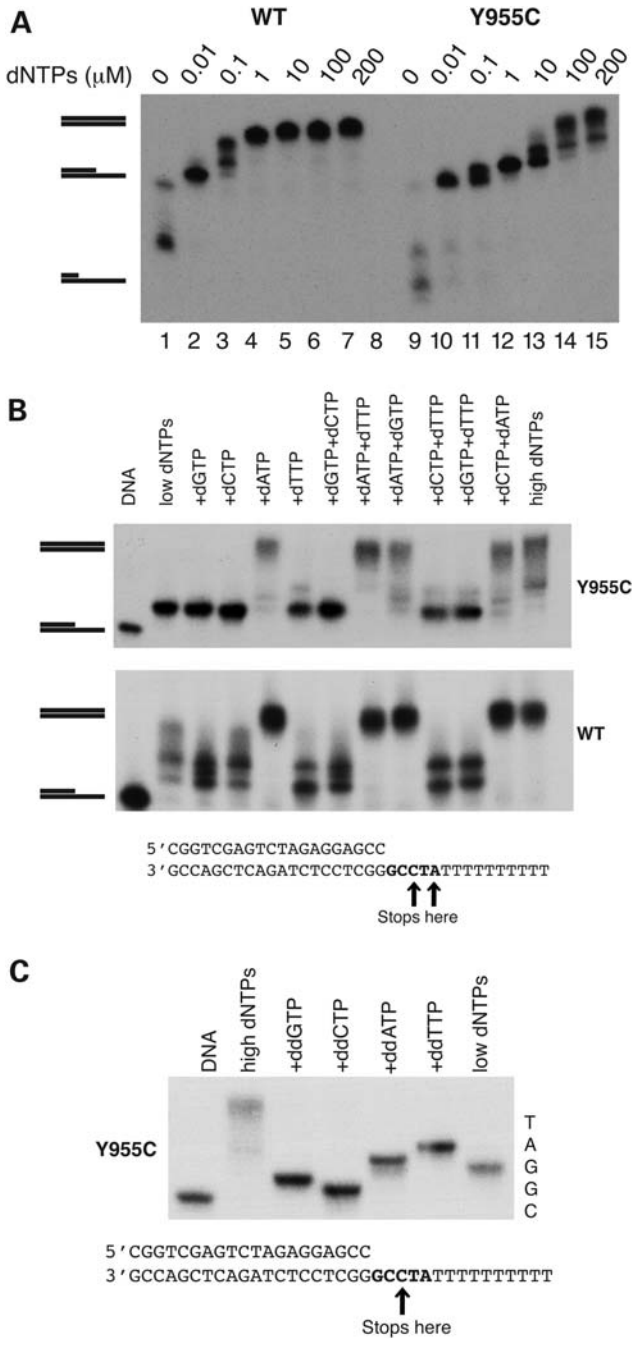

Figure 5. Both POLγA and POLγA:Y955C have a specific requirement for higher dATP concentrations. (**A**) A coupled DNA polymerase/exonuclease assay. The reaction was carried out as described in Materials and Methods, using a primed DNA template, 60 fmol of the indicated POLγA versions, 120 fmol of POLγB and the indicated concentration of dNTPs. The positions of the non-elongated primer (20-mer), the elongated primer (35-mer) and the degraded primer (10-mer) are indicated. (**B**) A stalling experiment was carried out as described in Materials and Methods using the same template as in (A). POLγA:Y955C (upper panel) and wild-type POLγA (lower panel) were incubated for 15 min at 37°C in the presence of low concentrations of dNTPs (1 μM dNTPs or 0.1 μM dNTPs, respectively). Higher concentrations of specific nucleotides (200 μM) were added to individual reactions, as indicated. (**C**) Mapping of the stalling site using the template described in (A). POLγA:Y955C was incubated with 1 μM dNTPs (lane 7) or 200 μM dNTPs (lanes 2–6). When indicated, one dNTP was replaced with the corresponding ddNTP.

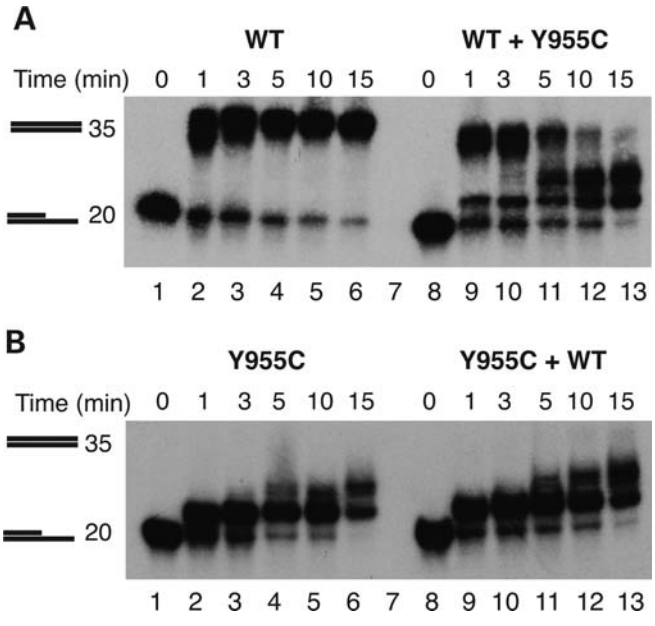

Figure 6. POLγA:Y955C actively breaks down already formed DNA products at low dNTP concentrations. Competition experiments were carried out as described in Materials and Methods using the same template as in Figure 5 and at constant concentrations of dNTPs (1 μM). In (**A**), wild-type POLγ (60 fmol of POLγA and 120 fmol of POLγB) was preincubated with the template on ice for 10 min, and three times higher amounts of POLγA:Y995C (and POLγB) was added and the samples were moved to 37°C for times indicated. (**B**) As in (A) but POLγA:Y995C was preincubated with the template on ice before wild-type POLγ was added.

As expected, POLγA:Y955C failed to complete synthesis even after 15 min of incubation at these dNTP concentrations (Fig. 6B, lane 6). We pre-incubated the template with the POLγA:Y955C protein for 10 min on ice, in order for the polymerase to bind the primed template. We next added the wild-type POLγ at a 1:1 ratio to POLγA:Y955C and transferred the reactions to 37°C to initiate DNA synthesis. We obtained results that were identical to those observed with POLγA:Y955C in isolation, supporting the idea that Y955C blocks the access to the primer template and demonstrating the dominant effect of this mutation.

We also performed the reverse experiments and pre-incubated wild-type POLγA with the template for 10 min on ice before addition of POLγA:Y955C. Interestingly, we observed effective, full-length DNA synthesis after 1 min as observed before with the wild-type POLγA in isolation, but upon longer incubation, the DNA synthesis products were progressively lost and at the end of the 15 min incubation, the reaction products were identical to those obtained with POLγA:Y955C in isolation (Fig. 6A, lanes 12–13). To our understanding, this is caused by an overlap of DNA synthesis and exonuclease activities: if loaded on a primer template, the wild-type POLγA can efficiently synthesize DNA in the presence of POLγA:Y955C. When DNA synthesis is completed, wild-type POLγA leaves the template and is replaced by POLγA:Y955C, which starts to chew back the poly-dA region of the template. When POLγA:Y955C reaches the end of the poly-dA region, the reaction stops and the polymerase is stalled in an idling mode. This

effect thus provides another explanation for the dominant mode of the POLγA:Y955C protein, since the protein may not only stall the replication fork in a site-specific manner, but also actively break down already formed DNA products.

DISCUSSION

We here demonstrate that the dominant and recessive nature of the Y955C and G848S mutations can be reconstituted *in vitro*. The G848S mutation severely decreases binding of POLγA to primed templates, but does not impair the balance between the DNA polymerase and exonuclease activities of POLγA. In agreement with its recessive nature in affected patients, wild-type POLγ appears unaffected by addition of POLγ:G848S. The POLγA:Y955C enzyme has an increased K_m for incoming nucleotides, and at dNTP concentrations sufficient for wild-type POLγA to function properly, POLγA:Y955C displays increased exonuclease activity, decreased processivity, and stalling phenotype. POLγA:Y955C competes with wild-type POLγ for binding to the primed template, but once the wild-type POLγA has commenced DNA synthesis, POLγA:Y955C does not affect the function of the replisome. The competition between POLγA and POLγA:Y955C leads to a heterogeneous population of short and long DNA replication products (Figs 2 and 3). POLγA:Y955C not only stalls during DNA synthesis, but can also bind to free 3′-ends and backtrack on DNA. Such backtracking may occur at nicks in one of the mtDNA strands or after wt POLγA has completed DNA synthesis, but prior to ligation (Fig. 7). Backtracking can thus destroy DNA synthesis products, leading to stretches of single-stranded gaps in the DNA. Single-stranded DNA regions are susceptible to strand breaks, which in turn may lead to deletions (25,26). These effects may therefore help to explain the dominant nature of the POLγA:Y955C mutation and also the high frequency of deletions seen in mtDNA isolated from patients with the Y955C mutation.

As demonstrated here, expression of POLγA:Y955C in cell culture leads to mtDNA depletion, whereas the same mutation causes an accumulation of deleted mtDNA molecules in tissues from affected patients. The reason for this discrepancy remains unclear, but the same phenomenon can be observed with other mutated versions of POLγA, and we have made similar observations with mutated forms of TWINKLE, expressed in cell culture or present in patient cell lines (20,27). We have extensively investigated cell lines expressing TWINKLE with adPEO-causing mutations and also cells expressing POLγA:Y955C, but have so far not detected any deletions. We have previously suggested a simple model to explain this inconsistency. In rapidly dividing cells, the mtDNA synthesis rate is much higher than that observed in differentiated tissues, and mutated versions of TWINKLE and POLγA may be unable to keep pace with the cell proliferation rate, thereby causing mtDNA depletion. This effect may not be seen in differentiated tissues, where mtDNA synthesis rate is much slower. We have previously suggested that mtDNA depletion may in fact occur, albeit transiently, in rapidly proliferating cells in a developing embryo and thus contribute to disease pathogenesis (26,27).

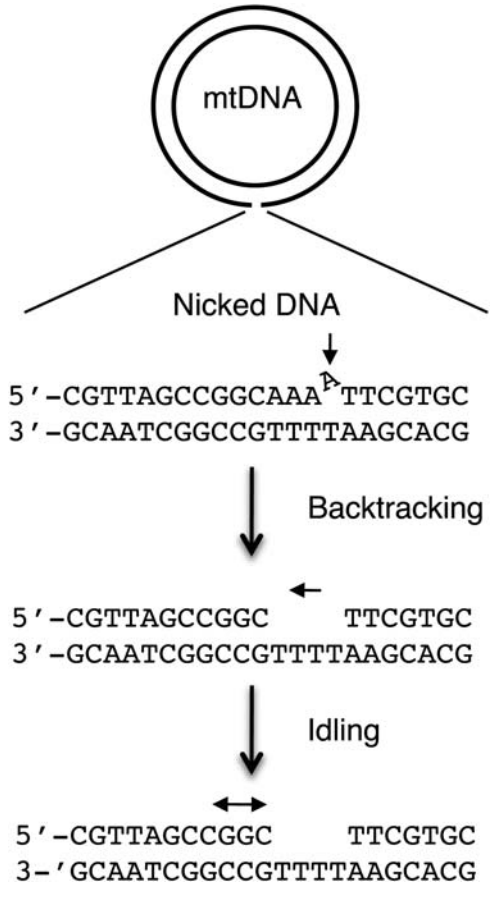

Figure 7. POLγA:Y955C displays increased exonuclease activity that can cause single-stranded gaps in mtDNA. POLγA:Y995C may generate single-stranded gaps in the DNA by binding to nicked mtDNA and backtracking. Since POLγA:Y955C is especially sensitive to dATP concentrations, the mutant enzyme may stall at dATP insertion sites and enter a polymerase/exonuclease idling mode.

The POLγ:Y955C mutation does not cause a significant increase in mtDNA point mutations in patients suffering from adPEO (8). In addition, direct measurements of the POLγ:Y955C error rate revealed only a 2-fold increase compared with the wild-type protein (10). In fact, when DNA polymerases make an error, they stall in order for the slower proofreading exonuclease to remove the mismatch (28). These observations are therefore in nice agreement with our observations here, demonstrating that POLγ instead of incorporating incorrect nucleotides enters an idling mode or backtracks. This effect may also help explain why POLγ:Y955C causes increased levels of mtDNA deletions, without affecting mtDNA mutations load, *in vivo*.

As demonstrated here, POLγA:Y955C is especially sensitive to low dATP concentrations and has an increased tendency to stall just before dATP insertions. Interestingly, wild-type POLγA is also extra sensitive to dATP levels compared with the other dNTPs, indicating that dATP levels are a critical factor also for wt POLγ processivity. The templates used in this report contain a single-stranded poly-dT stretch,

which may enhance the dATP dependency. We have however repeated the experiments with a mixed-sequence single-stranded stretch and observed a similar effect (Supplementary Material, Fig. S2C). Interestingly, we also noted a problem with dTTP insertion on this template (data not shown). This is in nice agreement with previous studies which have demonstrated that POLγ:Y955C also has a high K_m for insertion of dTTP (29). The Y955 residue is positioned within motif B in the finger domain and is part of the tyrosine couple Y951/Y955 that stacks and stabilizes the incoming dNTP and template base to allow base pairing to occur (23,28,30). The phenyl ring of Y955 stacks the template base, preventing it from wobbling, thus allowing base-pairing to occur with the incoming nucleotide. It is believed that there is a fast shuttling of the synthesized strand between the polymerase and exonuclease site, and the balance between the two activities is determined by the stability of the template base and the newly synthesized base (29). Since the dA–dT base pair is less stable than dG–dC base pair, the exonuclease activity will be more pronounced upon insertion of dATP or dTTP compared with the insertion of dGTP or dCTP.

AdPEO is not only caused by mutations in the DNA replication machinery, but also by mutations in the heart/skeletal muscle isoform of the adenine nucleotide translocator (ANT1) (31). In contrast to mutations in other factors regulating nucleotide metabolism within the mitochondrion, these mutations lead not to mtDNA depletion, but to mtDNA deletions in patients suffering from adPEO. Our findings here may explain this interesting difference. ANT1 is an abundant protein of the inner mitochondrial membrane and functions as a homodimer to create a channel through which ADP is transported into and ATP out of mitochondria (31). Our results show that mtDNA replication is very sensitive to dATP concentrations, which could explain why the ANT1 mutations give rise to deletions. The current knowledge does, however, not support dATP as a physiological substrate for ANT1. Nevertheless, mammalian mitochondria have been reported to contain ribonucleotide reductase required to reduce ADP to form dADP, which is phosphorylated to form dATP for DNA synthesis (32). However, this is a one-time observation and needs to be carefully re-addressed. It seems more likely that cytosolic ribonucleotide reductase activity in post-mitotic cells/tissues can quickly become rate limiting for mtDNA synthesis, as is illustrated by mutations in p53R2 that cause both mitochondrial depletion and multiple deletion syndromes (33,34). The results presented here suggest on the one hand that POLγ is a likely downstream effector of mtDNA disease-associated p53R2 mutations and on the other hand that POLγA:Y955C lowers the threshold at which cytosolic ribonucleotide reductase activity becomes detrimental to mtDNA metabolism.

Our observations correlate nicely with recent studies in yeast, which investigated the functional consequences of pathogenic mutations in the 3′–5′ exonuclease domain of Mip1, the mitochondrial DNA polymerase in *Saccharomyces cerevisiae* (35). These data demonstrated a close functional link between the exonuclease and polymerase domains of the protein and revealed that mutations in the 3′–5′ exonuclease module can impair the balance between DNA synthesis and degradation. In an analogous fashion, our studies demonstrate that also mutations in the polymerase domain of human POLγA may impair DNA synthesis and shift the balance between DNA synthesis and degradation, leading to mitochondrial disease in affected individuals.

MATERIALS AND METHODS

Recombinant proteins

TWINKLE, mtSSB, POLγA and POLγB were expressed and purified as described previously (16). A 6xHis-tagged version of POLγA cloned into pBac PAK9 was used for PCR-based mutagenesis. Plasmids containing G848S and Y955C mutations were sequenced and used to prepare *Autographa California* nuclear polyhedrosis virus recombinant for the proteins as described in the BacPAK manual (Clontech). The mutant versions of POLγA were purified following the same protocol used for the wild-type. We estimated the purity of the proteins to be at least 95% by SDS–PAGE with Coomassie blue staining (Fig. 1B).

In vitro DNA replication

The mini-circle template for rolling-circle DNA replication was generated as described previously (36). The mini-circle template (10 fmol) was added to a reaction mixture (20 μl) containing 25 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 100 μg/ml BSA, 4 mM ATP, 100 μM dATP, 100 μM dTTP, 100 μM dGTP, 10 μM dCTP, 2 μCi [α-^{32}P] dCTP and, if nothing else is indicated in the figure legends, TWINKLE (100 fmol), mtSSB (5 pmol), POLγA (100 fmol) and POLγB (150 fmol as a dimer). The reaction was incubated at 37°C and stopped at the times indicated by adding 200 μl of stop buffer [10 mM Tris–HCl (pH 8.0), 0.2 M NaCl, 1 mM EDTA and 0.1 mg/ml glycogen]. The samples were then treated with 0.5% SDS and 100 μg/ml proteinase K for 45 min at 42°C, and precipitated by adding 0.6 ml of ice-cold 95% ethanol. The pellets were washed with 70% ice-cold ethanol, dried at 45°C for 15 min and dissolved in 20 μl of water. Denaturing agarose gel electrophoresis was performed by taking 10 μl of each sample and adding 2 μl of alkaline loading buffer containing 18% (w/v) ficoll, 300 nM NaOH, 60 mM EDTA (pH 8.0), 0.15% (w/v) bromocresol green and 0.25% (w/v) xylene cyanol FF. The samples were loaded onto a 0.8% alkaline agarose gel (50 mM NaOH and 1 mM EDTA) and electrophoresis was carried out at 26 V for 20 h. The gels were dried onto DE81 (Whatman) and visualized by autoradiography overnight at −80°C with an intensifying screen.

Southern blot analysis

Rolling-circle reactions were carried out as described above, but without the addition of radioactive nucleotides and in the presence of the indicated concentration of each dNTP. The reactions were stopped after 40 min at 37°C by adding 6 μl of alkaline gel-loading buffer and 1 μl of 0.5 M EDTA and loaded onto a 0.8% alkaline agarose gel. After electrophoresis, the DNA was transferred to a nylonfilter

(N-Hybond+, Amersham Pharmacia). The membranes were hybridized with random-labeled probes from the mini-circle template produced using the random primer labeling kit (Stratagene).

Electrophoresis mobility shift assay

DNA-binding affinity of POLγA to a primer template was assayed using a 35-mer oligonucleotide (5′-TTT TTT TTT TAT CCG GGC TCC TCT AGA CTC GAC CG-3′) labeled in the 5′ end with [γ-^{32}P] ATP and annealed to a 20-mer complementary oligonucleotide (5′-CGG TCG AGT CTA GAG GAG CC-3′) to produce a primed template with a 15-base single-stranded 5′-tail as described previously (37). The reactions were carried out in 15 μl volumes containing 10 fmol DNA template, 20 mM Tris–HCl (pH 7.5), 1 mM DTT, 0.1 mg/ml bovine serum albumin, 10 mM $MgCl_2$, 10% glycerol, 2 mM ATP, 0.3 mM ddGTP and 3 mM dCTP. Proteins were added as indicated in the figure legends and reactions were incubated at room temperature for 10 min before separation on a 0.8% agarose gel in 1× TBE for 2 h at 100 V.

DNA synthesis assay

A 60-mer oligonucleotide (5′-GGC CCC CTA GGT GAT CAA GAC ACA TAA TTA TTC TTA TAA GAA CAT GTT CAT GCC GAG GTT-3′) was annealed to single-stranded pBluescript II KS+, which had been isolated according to the manufacturer's protocol (Stratagene). The template formed contains a 20 bp duplex region and a 40-base single-stranded 5′-tail. Reactions were carried out in 20 μl volumes containing 10 fmol template DNA, 10 mM Tris–HCl (pH 7.5), 1 mM DTT, 0.1 mg/ml BSA, 10 mM $MgCl_2$, 10% glycerol, 2 mM ATP, 10 μM dCTP, 100 μM dATP, 100 μM dTTP, 100 μM dGTP and 2 μCi [α-^{32}P] dCTP. Reactions were incubated at 37°C for the indicated times and separated on a 0.8% agarose gel at 120 V for 2 h in 1× TBE.

Exonuclease activity

To measure exonuclease activity, we annealed a 5′ ^{32}P-labeled 21-base oligonucleotide (5′-TGC ATG CCT GCA GGT CGA CTG-3′) to M13mp18 ssDNA, creating a 20-bp dsDNA region with a one-nucleotide mismatch at the 3′-end. The reactions were performed as described previously (38), but with 100 fmol of the indicated POLγA version and 300 fmol of POLγB. The products were analyzed by electrophoresis in 3 M urea/25% polyacrylamide gels.

Polymerase/3′–5′ exonuclease-coupled assay

A 20-mer (5′-CGG TCG AGT CTA GAG GAG CC-3′) labeled in the 5′-end with [γ-32P] ATP was annealed to a 35-mer oligo containing a poly-dT stretch, 5′-TTT TTT TTT TAT CCG GGC TCC TCT AGA CTC GAC CG-3′, or an oligo in which the poly-dT stretch had been replaced by a mixed sequence, 5′-GAC TAC GTC TAT CCG GGC TCC TCT AGA CTC GAC CG-3′, to produce a primed template that could be used as a substrate to investigate both DNA polymerization and 3′–5′ exonuclease activity. The reaction mixture contained 10 fmol of the DNA template, 25 mM Tris–HCl (pH 7.5), 10% glycerol, 1 mM DTT, 10 mM $MgCl_2$, 100 μg/ml BSA, 60 fmol of either wild-type, Y955C or G848S mutant POLγA and 120 fmol of POLγB and the indicated concentrations of the four dNTPs. The reaction was incubated at 37°C for 15 min and stopped by the addition of 15 μl of gel-loading buffer (98% formamide, 10 mM EDTA, 0.025% XC, 0.025% BromPhenolBlue). The samples were analyzed on a 15% denaturing polyacrylamide gel in 1× TBE buffer. Polymerization or 3′–5′ exonuclease activity was detected by, respectively, an increase or a decrease in the size (20-mer) of the 5′-labeled primer.

Brewer–Fangman 2D neutral/neutral agarose electrophoresis

Mitochondria were purified using cytochalasine B (Sigma-Aldrich) and nucleic acids were purified essentially as described (21). The obtained mtDNA was digested with *Hinc*II (Fermentas). The fragments were separated using neutral 2D-AGE after which the gels were blotted and hybridized with ^{32}P-labeled DNA probes. The migration of the *Hinc*II digest fragment (mtDNA nucleotides 13636–1006) was studied with a probe containing the mtDNA nucleotides 14846–15357.

Quantitative real-time PCR

The mtDNA content per cell was determined by real-time PCR of cytochrome *b* (cyt *b*) with the gene encoding the amyloid precursor protein *APP* as a nuclear standard as described (39). DNA extracts were obtained from cells by lysis, proteinase K treatment and subsequent isopropanol precipitation. The copy numbers of cyt *b* and *APP* were determined in a duplex Taqman PCR on an Abiprism 7000 (Applied Biosciences) using pCR® 2.1-TOPO® (Invitrogen) containing the cyt *b* and *APP* amplicon as standards.

Cell culture

Human cultured cells were grown in Dulbecco's modified Eagle's medium supplemented with 110 mg/l pyruvate and 10% fetal bovine serum. The POLγ wild-type and Y955C mutant with His tags were cloned into pcDNA5 and transfected into human embryonic kidney Flp-In TRex cells (Invitrogen), where expression of the transgene is under the control of a tetracycline/doxycyclin-inducible promoter. The obtained cell lines were verified for inducible expression of the transgenic protein by western blotting. To induce expression, the indicated amount of doxycycline (Sigma) was added to the growth medium.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

Conflict of Interest statement. None declared.

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