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Base excision repair in early zebrafish development: evidence for DNA polymerase switching and standby AP endonuclease activity

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Honors Thesis

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Finally, I would like to thank and dedicate this thesis to my parents, Pamela Cheyne and Michael Fortier. Thank you for supporting me in all my endeavors.

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Abbreviations

AP abasic site
BER base excision repair
dpf days post fertilization
ds double strand
hpf hours post fertilization
MBT midblastula transition
MO morpholino oligonucleotide
Pol β DNA polymerase β
qRTPCR quantitative real-time polymerase chain reaction
ROS reactive oxygen species
TS-MO morpholino oligonucleotide directed against the translation start site
UDG eukaryotic uracil DNA glycosylase
Ung prokaryotic uracil DNA glycosylase
ZAP1 zebrafish AP endonuclease 1 protein

Abstract

Oxidative damage to DNA is a byproduct of normal processes essential for life, and therefore subsequent repair is necessary to maintain cell vitality. Damage to DNA causes incorrect base pairing that can lead to nonfunctional proteins and consequently result in cell abnormalities, cancer or death. Base excision repair (BER) functions to repair non-bulky lesions, uracil, and sites lacking a nucleobase. The pathway can utilize one of two mechanisms known as short or long patch repair in which there is single or multiple base insertion respectively. BER begins with the recognition and removal of the incorrect or damaged base via a DNA glycosylase. The resulting apurinic/apyrimidinic site (AP site) is cleaved with AP endonuclease (AP endo). In short patch repair, a DNA polymerase mediates subsequent insertion of a single correct base and removal of the deoxyribose phosphate group (dRP). In long patch repair, a replicative DNA polymerase inserts 2-6 bases and the resulting displaced 3' strand is cleaved by flap endonuclease 1 (FEN1). Finally, the DNA strand is sealed with DNA ligase. Several of these key enzymes are embryonic lethals in knockout mice. Although BER is well characterized in adult tissues and cells, its role during development is poorly understood. Since the pathway has never been investigated during embryogenesis, we characterized BER in zebrafish extracts from unfertilized eggs, embryos at different developmental stages and adults. Using a 45-mer double-stranded substrate with a U/G mispair at position 21, we showed that extracts from all stages are capable of performing BER. Before 3 dpf aphidicolin-*sensitive* polymerases

perform most nucleotide insertion. After hatching at 3 dpf, an aphidicolin-*resistant* polymerase, probably DNA polymerase β, becomes the primary polymerase. Previously we showed that when zebrafish AP endonuclease protein (ZAP1) level is knocked down, embryos cease dividing after the initial phase of rapid proliferation and die without apoptosis shortly thereafter. Nevertheless, extracts from embryos in which ZAP1 has been largely depleted process substrate equally as well as extracts from control embryos. Since *apex1* and *apex2* are *both* strongly expressed in early embryos relative to adults, these data indicate that *both* may play important roles in DNA repair in early development. In brief, early stage embryos are fully capable of cleaving an AP site after substantial depletion of AP endo and of inserting repair deoxynucleotides despite a lack of detectable DNA polymerase β protein. Therefore, the major differences in BER performed by early stage embryos and adults are the predominance of replicative polymerases and the presence of backup Mg^{2+}-dependent endonuclease activity in early stage embryos. The switch to normal, adult BER occurs when embryos hatch from the chorionic membrane and encounter normal oxidative stress.

Introduction

Despite the importance of DNA repair in cellular physiology (1), relatively little is understood about the role of DNA repair in embryonic development. All cells make reactive oxygen species (ROS) as a by-product of ATP synthesis via the electron transport chain (2-4). Estimates predict that as many as 20,000 bases in DNA are damaged in each human cell per day by ROS. ROS damage proteins, lipids, carbohydrates and DNA. Although the first three can be discarded and resynthesized, the cell cannot simply discard its DNA without losing vital genetic information. Therefore, all organisms have a pathway known as base excision repair (BER) to repair oxidatively damaged DNA (Figure 1).

BER is initiated through cleavage of the glycosidic bond linking the aberrant base and the sugar-phosphate backbone. A family of enzymes known as DNA glycosylases catalyze this step by scanning the double helix for abnormal or modified bases. DNA glycosylases accomplish the cleavage step by flipping the nucleobase out of the helix into the active site where subsequent cleavage occurs (5). Hydrolysis of the glycosidic bond results in an AP site. The AP site is processed by AP endonuclease (AP endo). AP endo is a magnesium dependent enzyme that catalyzes the cleavage of the phosphodiester bond 5' to the AP site (6). This leaves a free 3' hydroxyl group and a deoxyribose phosphate (dRP) residue. The dRP residue must be removed in order to allow for eventual ligation. The aforementioned DNA product (substrate III in Figure

1) can serve as a substrate for either short or long patch BER. In adult short patch repair, base insertion and lyase activity are each performed by DNA polymerase β (pol β) (7). In adult long patch repair, PCNA-dependent replicative polymerases such as α, δ, and ε mediate the insertion of 2-6 nucleotides (8). An additional enzyme, flap endonuclease 1 (FEN1), is required to remove the overhanging flap of DNA bases (9). The resulting product from both repair pathways is sealed with DNA ligase I or III (10).

AP endo, XRCC1, FEN1 and ligase I are required for embryonic development in mice (11-15). When pol β is deleted, embryos exhibit a lethal phenotype (16) or abnormal neurogenesis and lung function, leading to neonatal death (17). Cell lines that are Polβ-/- are viable but hypersensitive to methylating agents such as methylmethanesulfonate (18-20). *Apex1 -/-* murine embryos survive no longer than embryonic day 9 (11, 12) and no homozygous null cell lines have been cultured to date (21). These studies underscore the importance of BER during development.

Although BER is crucial for normal development, it has not been described before fertilization and during embryogenesis. We have utilized zebrafish (*Danio rerio*) eggs and embryos to explore and characterize the BER pathway *in vitro*. The zebrafish serves as a model organism for studying development. As opposed to mammalian models such as the rat and mouse, zebrafish embryos develop rapidly and progress from eggs to larvae in less than three days (22). The embryos develop externally to the mother, which facilitates experimental manipulation and observation. They are well disposed to simple staining techniques and drugs that can be administered directly to

their surroundings (22). Moreover, their large egg size allows for easy microinjection of morpholino oligonucleotides (MO) to prevent targeted proteins from being translated (22, 23).

In an effort to understand the role of *Apex1* in early development, we recently explored the effects of knocking down expression of the gene in zebrafish embryos (24). Using MO to prevent translation of *Apex1*, we demonstrated that full knockdown results in death after the rapid phase of cell division is complete, following the midblastula transition (MBT). Although zebrafish zygotic transcription begins at the MBT, failure to initiate zygotic transcription is unlikely to be the cause of death, because mouse embryos initiate zygotic transcription at the two-cell stage but *Apex -/-* mouse embryos do not fail until gastrulation at E7-E9.

Zebrafish embryos in which translation of apex1 mRNA has been blocked by means of TS-MO (full knockdown) can be rescued through the MBT by co-injection of the mRNA for WT *human* Apex1, although development is still not normal and the embryos die at ~7 days after fertilization (dpf). The same phenotype arises in partial knockdowns (hypomorphs) if the protein is knocked down by less than 60% by means of lower concentrations of TS-MO or if the MO targets the splice sites of *apex1* (24). In an effort to examine whether full or partial knockdown of *apex1* results in loss of BER, we have examined the BER pathway (Figure 1) in extracts of eggs, early embryos and adult zebrafish and in full knockdowns and hypomorphic embryos at the appropriate stage of development. We find that eggs, embryos and adult zebrafish are capable of BER.

Even when ZAP1 (zebrafish AP endonuclease protein) has been reduced by >70%, embryonic extracts are still capable of repairing a U/G mispair to the same extent as extracts from controls. While adult mammalian cells have a single major AP endonuclease, encoded by *apex1*, here we provide functional evidence for the presence of additional Mg^{2+}-dependent AP endo activities in zebrafish embryos, most likely AP endonuclease 2.

Furthermore, we find that pol β does not mediate base insertion in early embryogenesis due to the lack of detectable protein in unfertilized eggs and embryos up to 6.5 hpf. Moreover, early base insertion is very sensitive to aphidicolin suggesting the role of replicative polymerases in base insertion before gastrulation. Thus we report that one or more aphidicolin-sensitive DNA polymerases mediate base insertion in zebrafish eggs and early embryos.

Materials and Methods

Zebrafish culture: Zebrafish were grown and embryos were spawned as described (22, 24). To prepare full knockdown and hypomorphic embryos, embryos were microinjected with TS-MO as described (24). (TS-MO sequence)

Preparation of extracts: In order to conduct *in vitro* assays for assessing the activity of BER enzymes in zebrafish it was necessary to prepare extracts from eggs, embryos throughout development, and adult fish. To obtain extracts for *in vitro* experiments, eggs and embryos were lysed in lysis buffer (50 mM Tris-HCl, pH 7.8, containing 200 mM KCl, 2 mM EDTA, 2 mM dithiothreitol, and 0.2% NP40, 40% glycerol) including multiple protease inhibitors (Complete Mini, EDTA-free protease inhibitors, Roche Diagnostics, Indianapolis, IN)(19). Adult fish were first ground in liquid nitrogen and then suspended in lysis buffer with protease inhibitors. Extracts were centrifuged at 13,000 rpm at 4°C to remove particulate material and dialyzed, using Slide-A-Lyzer cassettes, against a large volume of 1x HEPES buffer (50 mM HEPES, 0.1 mM EDTA) to remove low molecular weight materials. Normalization of protein concentration by mass was accomplished through Bradford assays. Each extract was diluted at three different concentrations and subjected to Bio-Rad Dye Reagent (Hercules, CA). Protein

concentrations were calculated based on a standard curve generated with known concentrations of BSA.

BER assay: The BER assay was performed as described by Singhal *et al*.(25) in a 50 μL volume. A reaction mix was prepared and equal volumes of mix were added to each sample containing an appropriate volume of sterile water. The volume of water depended on the volume of reaction mix and extract and was determined by subtracting these two volumes from 50. Unless indicated otherwise, the reaction mix included 50 mM Hepes, pH 7.4; 5 mM $MgCl_2$; 1 mM dithiothreitol; 0.1 mM EDTA; 2 mM ATP; 0.5 mM NAD; dATP, dTTP and dGTP at 20 μM each; 5 mM sodium phosphocreatine; 10 units of creatine phosphokinase; 120 nM of duplex oligonucleotide; and 40 μM of the fourth dNTP. Reactions were initiated by adding a volume of extract that equated to 10 μg of total protein unless otherwise specified. Reactions were incubated for 0.5 – 60 min at 22 °C and stopped by addition of EDTA to a final concentration of 83 mM. DNA was extracted with phenol-chloroform and precipitated with three volumes of chilled ethanol. Substrates and products were then resolved by means of denaturing polyacrylamide gel electrophoresis employing a 15% gel in the presence of 7 M urea and observed using phosphorImager analysis (6, 26).

In all cases we used a 45-mer double-stranded oligonucleotide with a U/G mismatch at position 21 (6, 26). Figure 1 illustrates each reaction, labeled 1-5. When uracil DNA glycosylase activity (Reaction 1) alone was measured, Substrate I, end-labeled at the 5' end by means of polynucleotide kinase (New England Biolabs, Beverly MA) and [γ^{32}P]ATP, was used, Mg^{2+} was replaced with 4 mM EDTA and the reaction was stopped either by phenol extraction or the addition of the UDG inhibitor ugi which was the kind gift of Dr. S. Bennett (U. Oregon). When Reactions 1 and 2 were measured, Substrate I was end-labeled at the 5' end and dNTPs were omitted from the reaction mix. When AP endo activity (Reaction 2) was measured individually, substrate was prepared as described (6, 26, 27). Briefly, the 5' end-labeled ds 45-mer was treated with Ung (1 unit/100 pmole U residues) for 30 min at 37 °C after which the Ung was heat inactivated at 70 °C for 5 min and the oligonucleotide was allowed to reanneal by slowly cooling to room temperature. When Reactions 1-3 were measured, Substrate I, end-labeled at the 5' end, was used and either dCTP alone or all 4 dNTPs were included in the reaction mix. When Reaction 3a or b was measured separately, Substrate 1 was not end-labeled. Instead, insertion of [α^{32}P]dCTP was measured directly by using unlabeled double stranded 45-mer and dCTP (Reaction 3a) or all 4 dNTPs (Reactions 3a and 3b). Preliminary experiments determined the amount of extract protein and time required for measuring each step in the pathway (Figure 2).

Quantitative real-time polymerase chain reaction (qRTPCR). Total RNA was isolated from 60 *Danio rerio* embryos at 3.5 hpf or from 100 mg of freeze-dried adult fish with TRIzol Reagent from Invitrogen (Carlsbad, California) according to the vendor's instructions. The RNA precipitate was resuspended in 150 µL RNase-free water and stored at –20°C. The first cDNA strand was synthesized from 1 µg total RNA in a 20-µL reaction using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Warrington UK) according to the manufacturer's manual. The cDNA was stored at –20°C.

For qRTPCR, the synthesized cDNA above was diluted 1:100 in RNase-free water and 4 µL was added into a 20-µL qRTPCR reaction mix containing SYBR Green PCR master mix (Applied Biosystems), 2 pmol each forward and reverse DNA primers, and water, then quantified in real time with an *ABI* PRISM® *7000* Sequence Detection System (Applied Biosystems) programmed thus: 1 cycle of 50°C for 2 min; 1 cycle of 95°C for 10 min; 40 cycles of (94°C for 15 s; 55°C for 30 s; 68°C for 60 s); 1 cycle of 68°C for 7 min, and 1 cycle of 4°C for 2 min. Dissociation curves were generated to ensure only one PCR product was obtained. Additionally, twelve PCR products were verified independently to contain only one product by agarose gel electrophoresis and ethidium bromide staining.

The messenger RNAs of the following genes were quantified by means of primers listed in Table 1: *apex1*, *apex2*, *bactin2*, *aprt*, *ef1a*, and *gapdh*. All measures of mRNA

quantity were performed in triplicate and the data were normalized to *gapdh* as the other genes gave $DC_T > 4$, indicating a greater than 16-fold differential in the level of expression of those genes in the 3.5 hpf embryo compared to the adult fish. *Gapdh* is preferred over *bactin2* for normalization of gene expression in zebrafish (28).

Western blots: Western blots were performed as described using rabbit polyclonal antibody prepared against ZAP1 in this laboratory (24), mouse monoclonal anti Arabidopsis β-actin (GeneTex, Inc., San Antonio TX), mouse monoclonal anti rat pol β (Thermo Fisher Scientific, Freemont CA), or rabbit polyclonal antibody prepared by 21rst Century Biochemicals, (Marlboro MA) against a peptide comprising amino acids 324 - 339 (FEYIQWKYREPKDRSE) of zebrafish pol β.

Results

Optimization of 45-mer oligonucleotide substrate and dCTP concentrations. Adult extracts were used as a means to determine the optimal concentrations of oligonucleotide and dCTP. As seen in Figure 2 (panel A), varying concentrations of 45-mer oligonucleotide were added to each sample containing 1 μM of dCTP. dCTP incorporation was measured using [α^{32}P]dCTP. A concentration of 120 nM of oligonucleotide optimized [α^{32}P]dCTP incorporated product. To assess the optimal concentration of dCTP, 1 μCi of [α^{32}P]dCTP was added to each sample with varying concentrations of unlabeled dCTP containing 120 nM oligonucleotide (panel B). A concentration of 40 μM of unlabeled dCTP optimized [α^{32}P]dCTP incorporation.

Extracts from eggs and adult fish are capable of performing BER. The individual steps in the BER pathway are outlined in Figure 1, which also details the substrates used to measure the entire pathway or individual steps. The choice of a substrate with a U/G mispair restricts the results to a uracil glycosylase-initiated BER cascade and minimizes the likelihood of confounding the data with pathways involving polynucleotide kinase and NEIL1 (29). In the first series of experiments we investigated whether extracts from eggs and adults could cleave a 45-mer ds substrate containing a U/G mispair at position 21 (Substrate I) and then insert the correct base opposite the orphan G residue. Loss of the 45-mer substrate and appearance of the 20-

mer product are shown in Figure 3, Panels A and C. Cleavage represents both removal of the uracil residue (Reaction 1) and also cleavage by ZAP1 (Reaction 2). The same experiment was also performed in the presence of dCTP (Panels B and D), which is incorporated into the cleaved upstream strand (Reaction 3a). Appearance of the 21-mer in the presence of dCTP represents the first two reactions followed by insertion of deoxycytidine (21-mer, Reaction 3a). Extracts from both eggs and adults (10 μg protein from extract) were capable of removing uracil from a U-containing double stranded 45-mer, cleaving the resulting AP-site and inserting a deoxycytidine opposite the orphan G. However, the rate of cleavage followed by insertion was less in eggs than in adults. Since the level of exonuclease activity increased markedly after 15 min, subsequent assays were performed over 15 min intervals or less. Examination of individual reactions provided the rationale for the differences, as shown in the following sections.

Extracts from eggs, embryos at different stages of early development and adults recognize and remove a U residue in DNA by means of uracil DNA glycosylase (UDG). Cleavage of the initial 45-mer requires successive activities of a uracil DNA glycosylase (UDG) and an AP endonuclease. To understand the slower cleavage rate on the part of egg extracts compared to extracts from adult fish, we examined each step individually. To observe the appearance of the AP-site in the substrate, we added EDTA to the reaction mix to a final concentration of 4 mM to inhibit divalent cation-

dependent reactions that might cleave the AP-site, and $NaBH_4$, which stabilizes the AP site as it forms but does not inhibit UDG or interfere with subsequent endonuclease cleavage (27, 30). In order to confirm that the uracil-removal was due to UDG, we examined to what degree Ugi (31) was able to inhibit the reaction and also the degree of enhancement on a single stranded substrate, which is a characteristic of UDG. We then subjected the purified products to cleavage with recombinant human AP endonuclease, which does not cleave the substrate unless an AP site is present. Figure 4 illustrates that extracts from all stages of development were able to remove uracil from the 45-mer and that the activity was entirely inhibited by Ugi. Uracil removal in embryos and eggs was ~60% more efficient on a single-stranded substrate than on a double-stranded substrate, which is characteristic of UDG (32). Extracts from adult fish, however, were not more active on a single stranded substrate, which could imply the presence of additional Ugi-sensitive glycosylases. Alternatively, there may exist additional nucleases active on a single-stranded oligonucleotide in extracts from adult fish that are not present in eggs and early embryos. Since the rate of removal of uracil from a double-stranded substrate in egg extracts was about half that in adult extracts, it is likely that part of the diminished activity in BER steps 1 and 2 seen in egg extracts was due to diminished processing of substrate at Step 1 in egg extracts.

Extracts from eggs, embryos at different stages of early development and adults cleave an AP-site efficiently. ZAP1 is present in eggs and throughout development (24). To avoid complications that might arise from reactions preceding the cleavage of the AP-site, we used a substrate with the same reduced AP site employed for examining the kinetics of human AP endonuclease (6, 26). This substrate allowed us to answer questions about the efficiency of AP-site cleavage by extracts from eggs, embryos and adults. The time course was linear for ~ 2 min when 2 μg protein from either adult or egg extracts was added to the mix, as shown in Figure 5A. Using the 1 min time interval, all extracts were able to cleave an AP-site containing substrate (Figure 5B). However, extracts from unfertilized eggs had about 1/4 the activity of extracts from adults, while embryos less than 72 hours after fertilization (hpf) had about half the activity of adult extracts. We showed earlier that the protein level of ZAP1 is constant throughout early development even as cellular proliferation continues (24). Nevertheless, the diminution in efficiency of the first three steps in the BER pathway in extracts from eggs as opposed to adults was due to diminished entry into the pathway via UDG and less efficient AP endonuclease *activity* in extracts from the former.

Knockdown of ZAP1 levels does not alter BER in early embryogenesis. Microinjection of MO into early embryos is a standard way of knocking down protein levels in zebrafish (22). The degree of knockdown varies with the amount of

microinjected MO. In this series of experiments we knocked down ZAP1 levels by 74% or 54%, as shown by Western blot analysis (data not shown) and examined both the ability to cleave an AP-site (Figure 5, 3.5* and 6**) and the time course of the first three steps in BER (Figure 6). Extracts were prepared from control and full knockdown embryos just after the MBT at 3.5 hpf (3.5*); extracts were also prepared from control and hypomorphic embryos at 6.5 hpf (6**) several hours after the MBT. Despite loss of much of ZAP1 protein in full knockdowns and somewhat less in hypomorphs, we saw no difference in the first three steps of BER in comparison with controls nor was there any change in the ability to cleave an AP site.

Another AP endonuclease is expressed in very early stage embryos. (Experiments conducted by Richard A.O. Bennett) Since suppression of translation of ZAP1 did not result in complete loss of the protein, there may still have been sufficient ZAP1 in hypomorphs and full knockdown embryos to provide endonuclease activity for BER. Nevertheless, the possibility remained that another AP endo might be expressed during development. A potential candidate is AP endonuclease 2, whose enzymatic activity in humans is 1% that of its paralogue (33, 34) but which is involved in processing of AP sites during immunoglobulin class switching (35, 36). Therefore, we chose to examine whether AP endonuclease 2 might be expressed. Using *gapdh* as the standard of normalization, qRT-PCR, revealed that *both* AP endonuclease 1 *and* 2 are expressed in early stage embryos at a level 13 and 20-fold that of adults respectively

(Figure 7). Therefore, cleavage of an AP-site by extracts of unfertilized eggs and early embryos could be the result of both proteins.

Before hatching at 72 hpf, replicative polymerases predominate in BER. Pol β is the preferred polymerase for insertion of a single nucleotide during BER of cultured cells and tissues from adult animals (19, 20, 25). During long patch repair, either pol β, or the replicative polymerases α, and δ/ε together with PCNA may participate (37, 38). The presence in the reaction mix of the single nucleotide dCTP limited the reaction to short patch repair, whereas the presence of all four dNTPs enabled progression of the alternative long patch pathway. In order to examine the preferred polymerases during short patch (single nucleotide insertion) and long patch (insertion of 2-6 nucleotides) BER during development, we examined whether aphidicolin or dideoxyCTP could inhibit nucleotide insertion. Inhibition by aphidicolin is diagnostic for participation of the replicative polymerases α, δ and ε (39), while inhibition by ddCTP is considered diagnostic for pol β (25) (Figures 8 and 9). Because pol β has no proof reading ability, it readily inserts but cannot remove ddCTP, which then prevents further chain elongation or ligation.

In extracts from egg and early stages of development single nucleotide insertion was far more sensitive to inhibition by aphidicolin than 24 hpf or older embryos and adults (Figure 8). Neither the ability to insert a single nucleotide nor the sensitivity to

aphidicolin was altered by *apex1* knockdown. By 24 hpf, aphidicolin's ability to reduce insertion had decreased. At this time the drug decreased incorporation by only 33% from control values, indicating a shift towards insertion by a polymerase resistant to aphidicolin. After hatching (3 dpf) the rate of insertion even exceeded levels in extracts from adult fish. Finally, in extracts from adult fish aphidicolin was able to decrease single nucleotide insertion by only ~15%. This last level was consistent with aphidicolin's effect on BER (37, 39, 40) and with the participation of pol β in both long and short patch repair (41) from a variety of adult tissues and from many cultured cell lines.

Comparison of repair intermediates by extracts obtained from adults and unfertilized eggs in the presence of all four dNTPs using a 5'-end labeled substrate enabled us to confirm and extend the results obtained in the presence of dCTP alone (Figure 9). Despite the presence of exonuclease activity, the incubation time was continued to 60 min in order to better measure long patch repair. Figure 9 illustrates the involvement of replicative polymerases in both short and long patch repair as shown by sensitivity to aphidicolin in extracts from unfertilized eggs but not in extracts from adult fish. Figure 10 also shows the relative insensitivity to dideoxyCTP in long patch repair performed by egg extracts, confirming the surprisingly diminished involvement of pol β in BER performed by extracts from eggs.

Pol β protein does not appear until after the midblastula transition. (Experiments conducted by Xiaojie Yang) The use of aphidicolin-dependent polymerases in BER in the unfertilized egg and very early embryogenesis could be due to replicative polymerases outcompeting pol β for the cleaved AP site or to a lack of pol β. Therefore, we performed Western blot analysis on embryonic extracts obtained from unfertilized eggs and from embryos at different stages of development. Figure 10 represents data showing that pol β was not detectable in unfertilized eggs and very early stage embryos. However, the amount of pol β relative to β actin reached adult levels by 13 hpf. Therefore, the sensitivity to aphidicolin in unfertilized eggs and very early embryogenesis can be attributed to a lack of pol β.

Discussion

Here we have presented the data on the first three steps of BER in early zebrafish embryogenesis. Cell-free extracts from unfertilized eggs and early stage embryos as well as from adult fish are capable of recognizing and removing uracil in a U/G mispair by uracil DNA glycosylase, cleaving the resulting AP-site, and inserting the correct cytosine residue either during short patch or long patch repair. The overall pathway is less efficient in extracts from unfertilized eggs and early stage embryos. For steps 1 and 2 of the pathway, extracts from unfertilized eggs and very early stage embryos have ¼ - ½ the enzymatic activity of UDG and ZAP1 found in extracts from adult fish, which contributes to the lower rate of deoxynucleotide insertion in short patch synthesis. The difference lessens at ~3 dpf when hatching has occurred. This observation is consistent with the role that BER plays in repairing oxidatively damaged DNA. After hatching, the embryo is no longer protected by the chorionic membrane and must be prepared for exposure to whatever conditions it encounters in the environment.

Once a zebrafish egg is fertilized, it undergoes ten rapid division cycles to form the blastula in less than 3 hours (22). The preponderance of replicative polymerases in both short and long patch repair in eggs and very early stage embryos could be explained by the large amounts of these enzymes stored in preparation for the rapid cell division that follows fertilization. At the midblastula transition cell division slows,

zygotic transcription begins, spatial differentiation arises and the cells become motile. The egg stockpiles all the components necessary for the initial rapid cell division including DNA polymerases and accessory proteins, dNTPs and the mitochondrial systems to regenerate the required ATP. However, ATP generation is inevitably accompanied by ROS production with the concomitant damage to DNA (2-4). Therefore, the BER pathway is an important component in early embryogenesis.

The reduction in ZAP1 levels by 74% in full knockdown embryos leads to little, if any, reduction in BER activity. Although the remaining protein may be sufficient to provide endonuclease cleavage, there is still the possibility that a backup activity is present in eggs and early stage embryos. To our surprise there is 20 times the *apex2* message in early stage embryos as in adults. In fact, mRNA of both *apex* genes is expressed at higher levels in the early stage wild type embryos in comparison to adult fish. Thus, AP endonuclease 2 with less than 1% the activity of AP endonuclease 1 on a molar basis may play an important role in early embryological development. The former has recently been shown to participate in processing of AP sites during class switching in lymphocyte maturation, an essential process for immunological development (35).

Although the pol β transcript is expressed in early development (XJ Yang, Unpublished results), pol β *protein* appears in detectable amounts only after 6.5 hpf. Thus, aphidicolin-sensitive replicative polymerases provide a large majority of the insertion activity in BER in unfertilized eggs and early stage embryos through gastrulation.

Therefore we have provided functional evidence for both a standby endonuclease activity and qualitative differences in DNA polymerase activity in eggs and embryos before 3 dpf as compared to adults.

It is well known that BER is essential for cell viability in multicellular organisms and has been highly conserved throughout evolution (1). Both exogenous factors and endogenous byproducts of metabolism have provided strong selective pressures influencing the pathway's development while favoring its permanence. Understanding the BER pathway during zebrafish embryogenesis may provide insight into the sources of congenital defects and genetic predispositions that arise during development. Some genetic defects are linked to flaws in other DNA repair pathways such as xeroderma pigmentosum in nucleotide excision repair (1) and nonpolyposis colorectal cancer in mismatch repair (42). Given that this is the first work on BER in early zebrafish development, our data provide a foundation for further research into the early mechanisms that define BER and its role in normal development.

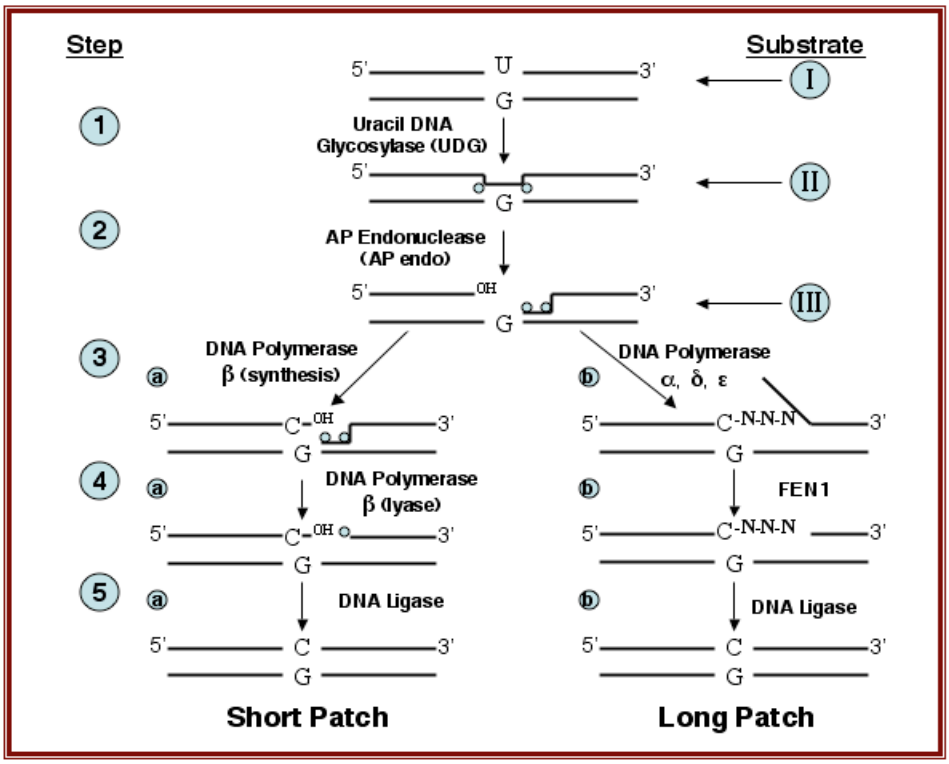

Figure 1. **The DNA base excision repair pathway** Step number is indicated on the left in Arabic numerals, while substrate used in different reactions shown in the text is indicated in Roman numerals on the right. Steps 3a, 4a and 5a occur in short patch (single nucleotide) repair. Steps 3b, 4b and 5b occur in long patch repair (insertion of 2-6 additional nucleotides). Step 3b uses the replicative DNA polymerases α, δ and ε, in the presence of PCNA (not shown).

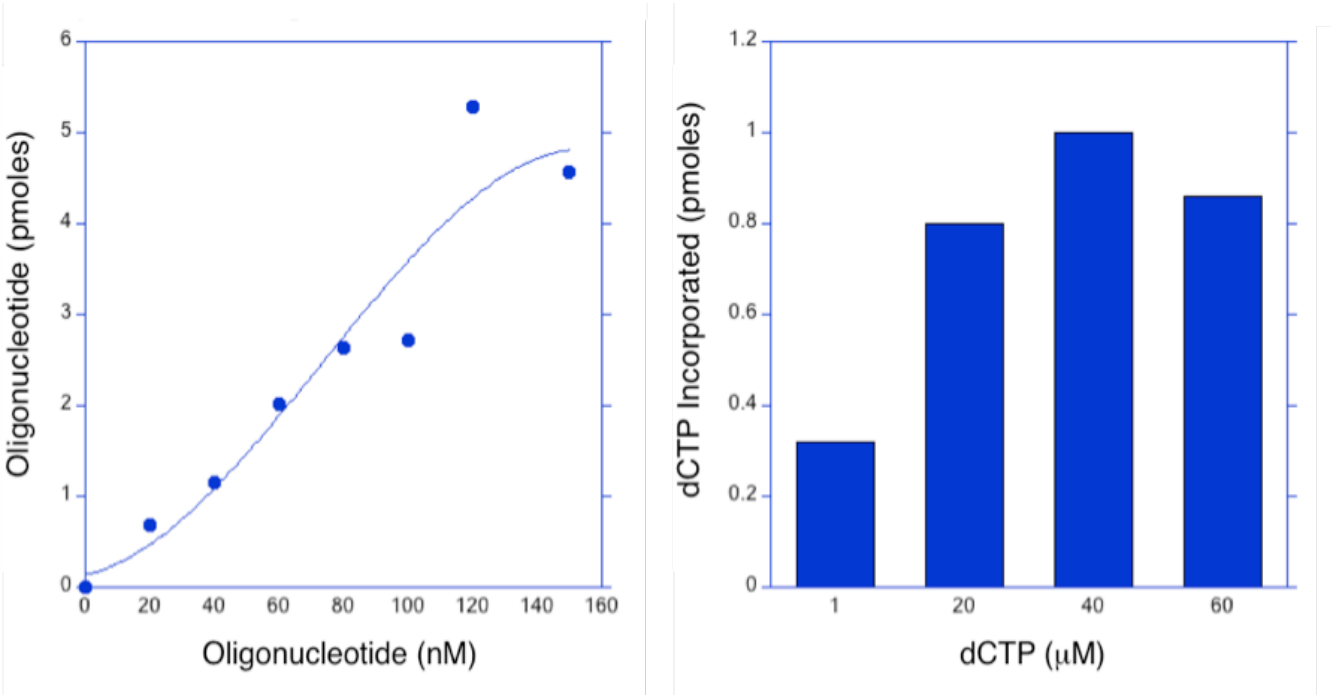

Figure 2. Optimization of 45-mer oligonucleotide substrate and dCTP concentrations. **A.** Adult extract (10 μg protein) was mixed with varying concentrations of Substrate I (45-mer containing a U/G mispair at position 21) in the presence of 1 μM dCTP and incubated for 90 min. **B.** Adult extract (10 μg protein) was mixed with 120 nM of substrate I and varying concentrations of unlabeled dCTP and incubated for 90 min. In each case, dCTP incorporation was measured using [α^{32}P]dCTP. After phenol extraction, products were resolved by denaturing polyacrylamide gel electrophoresis in the presence of urea and visualized by phosphorImager analysis. These data represent a single experiment.

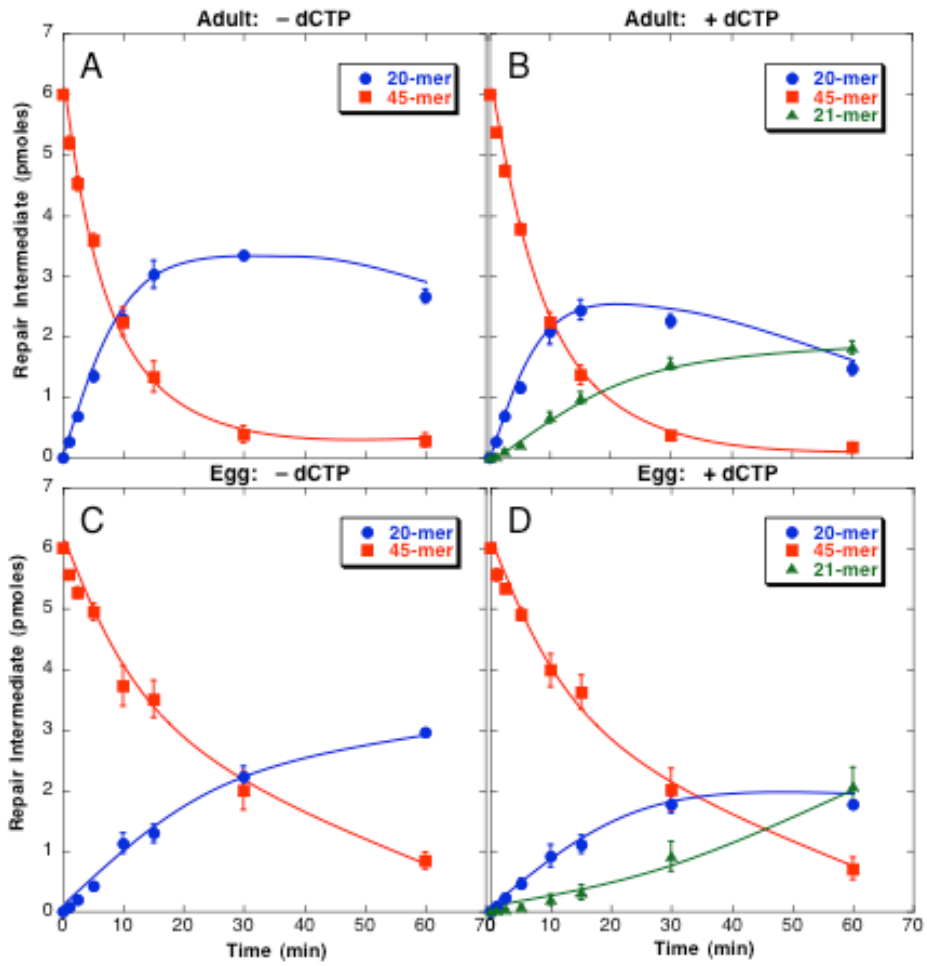

Figure 3. **BER in extracts from adult fish is more efficient than in extracts from eggs.** Extract (10 µg protein) from either adult fish (panels A and B) or unfertilized eggs (panels C and D) was mixed with Substrate I (45-mer containing a U/G mispair at position 21) in the absence (panels A and C) or presence of dCTP (panels B and D) for the length of time indicated. After phenol extraction, substrate and products were resolved by denaturing polyacrylamide gel electrophoresis in the presence of urea and visualized by phosphorImager analysis. These data represent an average of three experiments +/- SE.

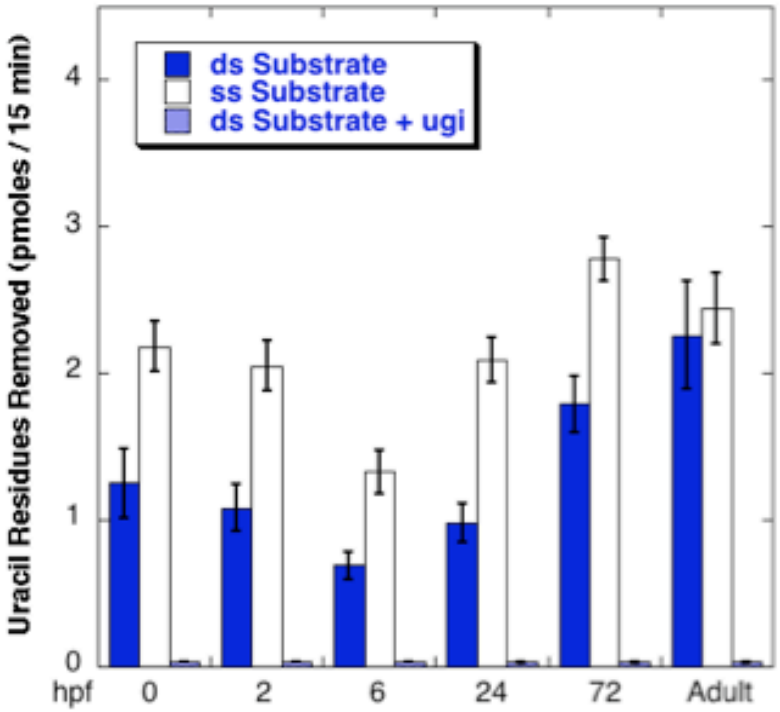

Figure 4. Uracil removal is performed by uracil DNA glycosylase, as shown by complete sensitivity to the UDG inhibitor Ugi. Extracts of eggs (0) and developing embryos at the indicated stages were incubated with 5'end-labeled 45-mer substrate in the absence of added Mg^{2+} and the presence of 4 mM EDTA for 15 min. Substrate was either double stranded (blue bars) or single stranded, lacking its complement (white bars). Double stranded substrate was also incubated in the presence of the UDG inhibitor, Ugi. The reaction was stopped by phenol extraction. After ethanol precipitation and resuspension in TE buffer, the samples were treated with human AP endo to cleave AP sites formed by the removal of uracil. There was no degradation of control substrate incubated in the absence of extract and processed in the same fashion. These data represent an average of three experiments +/- SE.

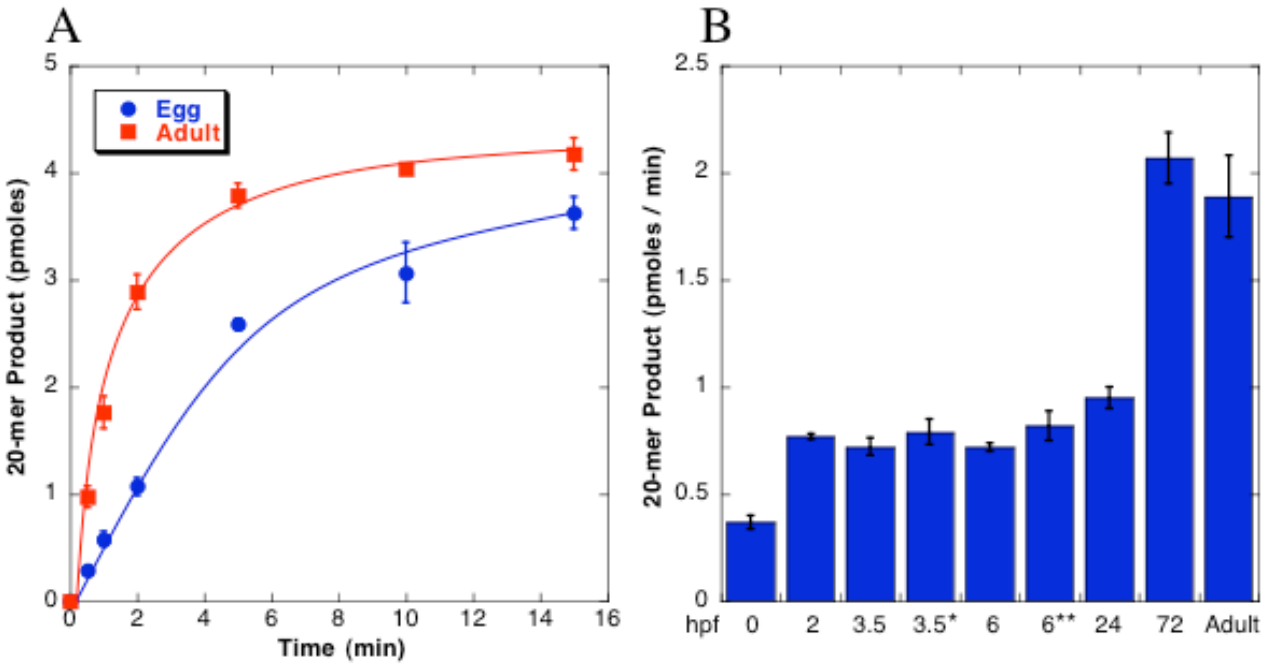

Figure 5. **AP site cleavage is inefficient in eggs and less efficient in pre-hatching embryos than at later times. A.** Extracts (2 µg protein) from eggs or adult fish were incubated for the indicated time in the presence of 5 mM Mg^{2+} with 5'-end labeled substrate that had been treated with Ung to remove uracil (21). Reactions were stopped by addition of EDTA, and phenol extracted. Substrate and product were resolved by gel electrophoresis. The rate of AP site cleavage was then examined in extracts (2 µg protein) obtained from eggs (=) or adult fish (<). AP site cleavage was linear for ~2 min under these conditions. **B.** AP site cleavage by eggs, embryos and adult fish (2 µg protein) over a 1 min interval. *: 3.5 hpf embryos in which ZAP1 has been reduced by 74-90 % by microinjection of 0.5 mM TS-MO at the 2-4 cell stage; **: extracts from 6.5 hpf embryos in which ZAP1 has been reduced by 40-56 % by microinjection of 0.2 mM TS-MO. These data are the average of three experiments +/- SE.

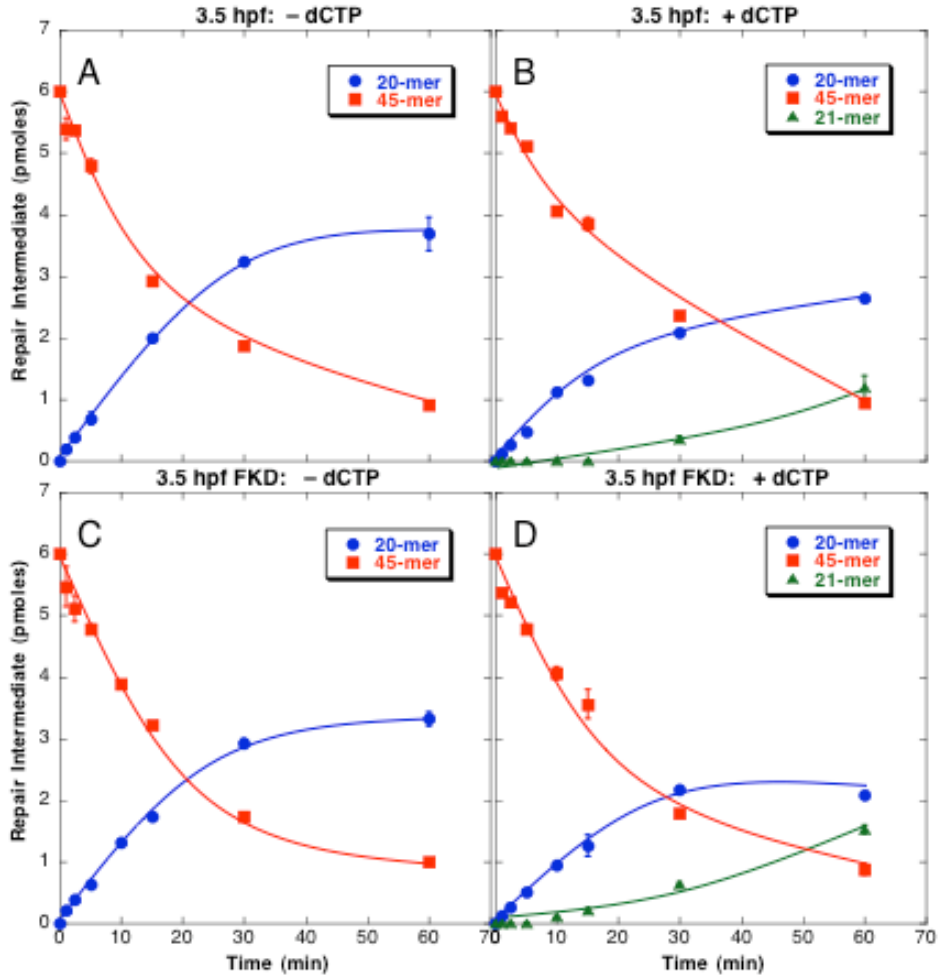

Figure 6. Time dependent processing of 45-mer substrate is not altered by diminution of ZAP1. These experiments were performed as described in the legend to Figure 3 except that extracts were obtained from 3.5 hpf control (A and B) or full knockdown (FKD) (C and D) embryos.

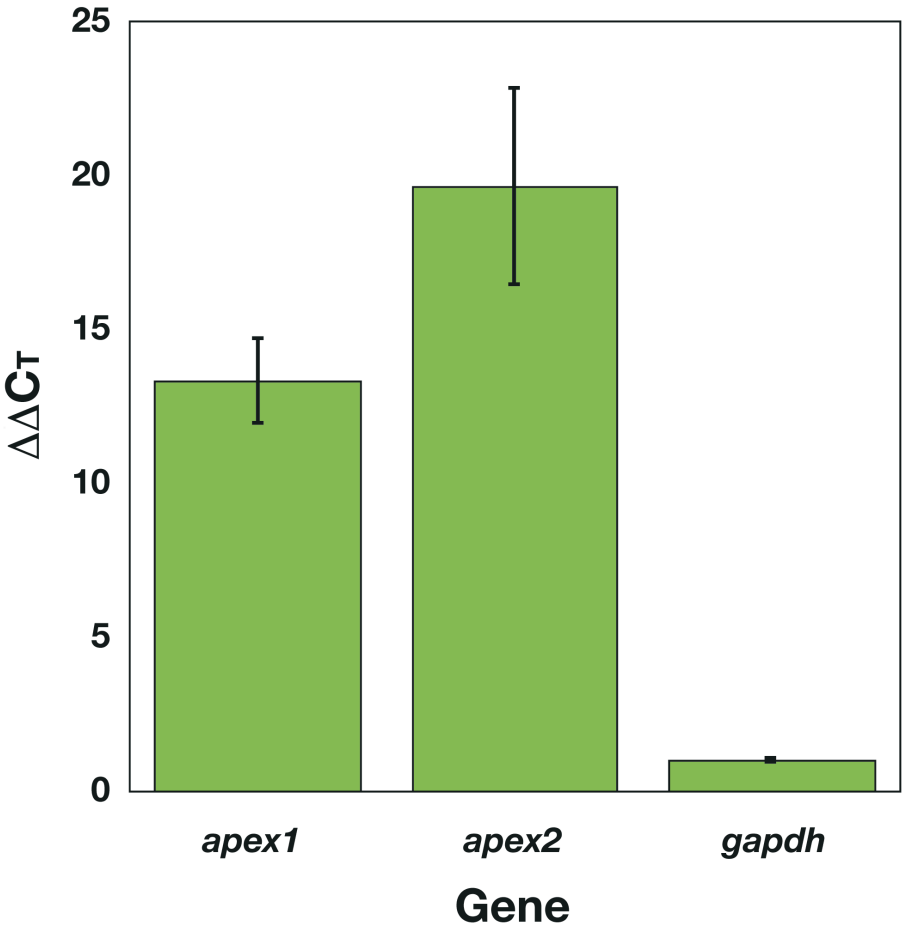

Figure 7. AP endonuclease 2 may serve a redundant function for AP endonuclease 1 during very early development. (Experiments conducted by Richard A.O. Bennett) qRTPCR was performed to examine expression levels of *apex1*, *apex 2* and *gapdh* in early stage, untreated embryos (3.5 hpf). ΔC_T values were calculated by normalization to *gapdh* for the embryo and adult fish. Relative gene expression (plotted), $\Delta\Delta C_T$, was calculated by subtracting the ΔC_T of the adult from the ΔC_T of the embryo for *apex1*, *apex2*, and *gapdh*. Data are means of triplicate values calculated from at least one primer pair for each gene over two independent experimental runs. Error bars indicate standard error of the mean.

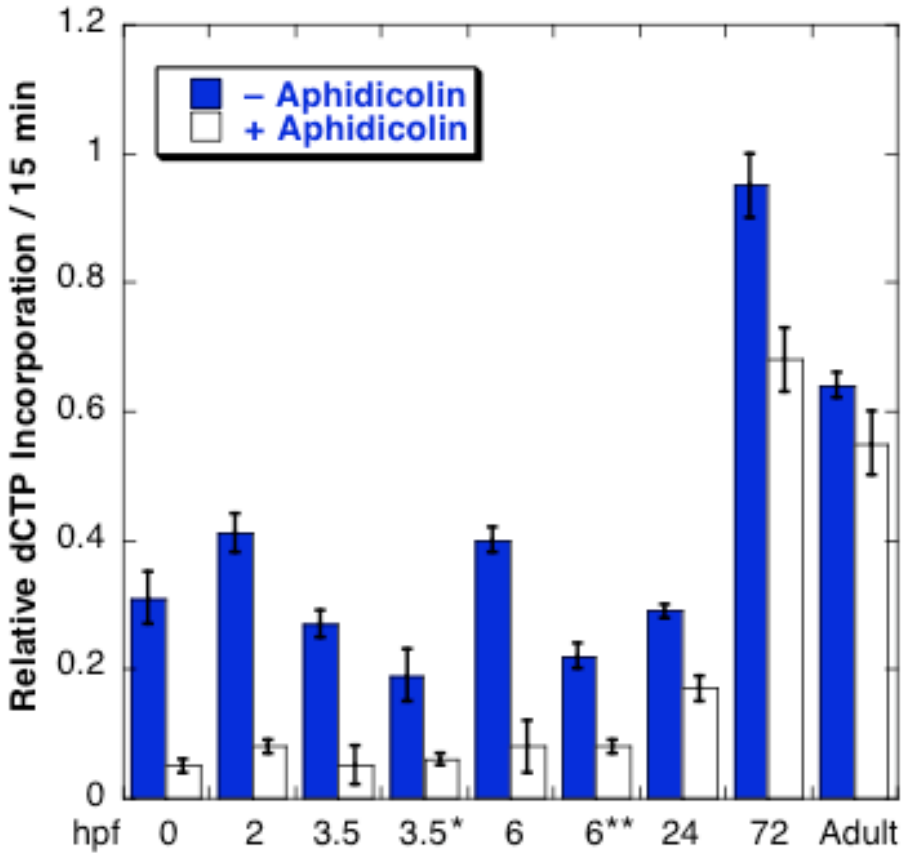

Figure 8. The bulk of short patch repair before hatching (72 hpf) is mediated by replicative polymerases. Extracts from eggs, embryos at various stages and adult fish were incubated in the presence or absence of aphidicolin (40 μM) with 45-mer substrate, which had been treated with Ung and human AP endo to generate the free 3' hydroxyl group capable of accepting a new incoming nucleotide. The incubation medium included [α^{32}P]dCTP. Insertion of dCTP was measured after phenol extraction and resolution of substrate and product by denaturing gel electrophoresis in the presence of 7 M urea. These data are the average of two experiments +/- range. Solid bars: - aphidicolin; open bars: + aphidicolin.

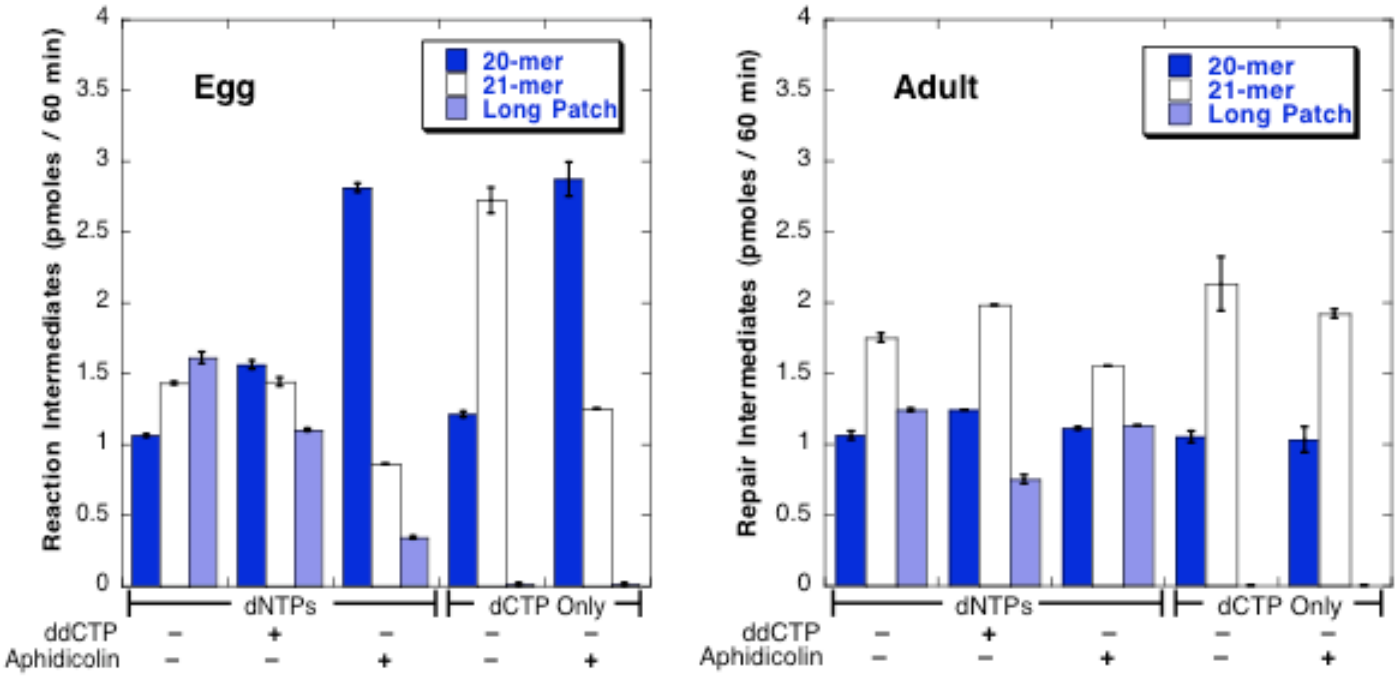

Figure 9. Unlike synthesis in adult extract, both short patch and long patch insertion in egg extracts is mediated by replicative polymerases. For this series of experiments, substrate was 45-mer oligonucleotide end-labeled at the 5' end of the U-containing strand. Extracts from unfertilized eggs or adult fish were assayed in the presence or absence of 40 μM aphidicolin or 400 μM dideoxyCTP for products formed over a 60-min interval in the presence of all 4 dNTPs or dCTP only in the presence or absence of aphidicolin or dideoxyCTP. Dark blue bars: 20-mer; clear bars: 21-mer; light blue bars: total 22-45-mer.

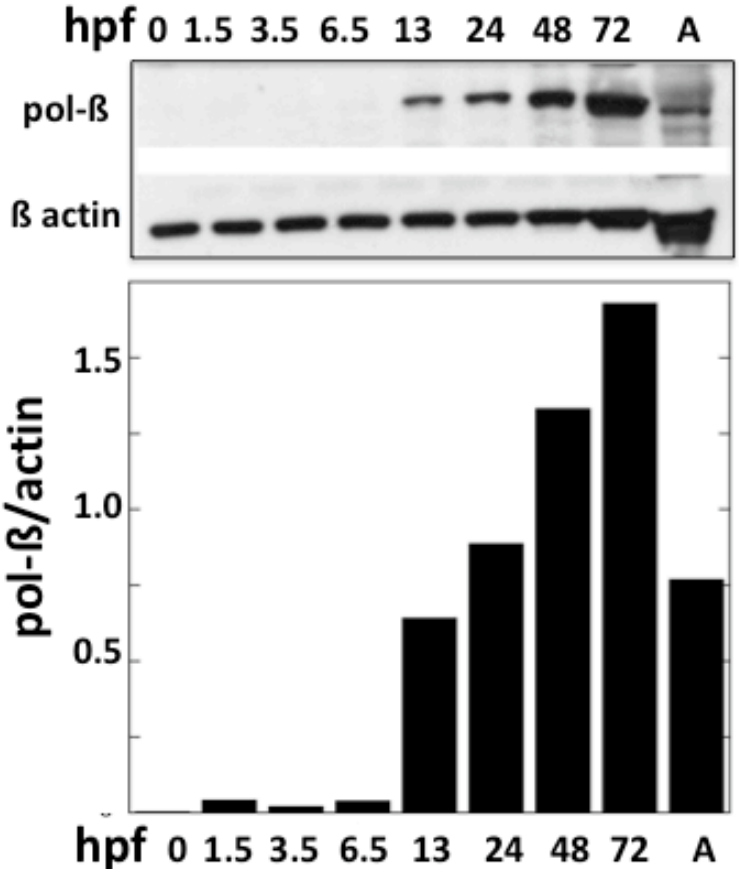

Figure 10. Pol β is not detected in unfertilized eggs and very early stage embryos. (Experiments conducted by Xiaojie Yang) Protein (100 μg/lane) in extracts of unfertilized eggs and embryos at various stages was resolved by SDS-PAGE, transferred to membranes and probed with mouse anti-rat pol β. The same extracts (40 μg/lane) were used for preparing membranes probed with mouse anti β actin. Western blot results are shown in the upper panel. The ratio of pol β/β actin at each time is shown in the chart. Time after fertilization when embryos were harvested (hpf) is indicated above the Western blot and below each lane in the bar graph. A, extract prepared from adult fish.

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