Arrested DNA Replication in *Xenopus* and Release by *Escherichia coli* Mutagenesis Proteins

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Xenopus oocytes and oocyte nuclear extracts repair ultraviolet photoproducts on double-stranded (ds) DNA and replicate single-stranded (ss) to ds DNA. M13 ss DNA molecules containing cyclobutane pyrimidine dimers were maintained but not replicated in *Xenopus* oocytes, yet were replicated in progesterone-matured oocytes. The replication arrest functioned only in cis. The replication arrest was alleviated by injection into oocytes of messenger RNAs encoding the prokaryotic mutagenesis proteins UmuD′C or MucA′B. These results may help explain how cells stabilize repair or replication events on DNA with unrepairable lesions.

Several types of DNA damage trigger cell cycle arrest, presumably to allow DNA repair to occur before the cell enters the S phase or begins apoptosis (*1*) when damage is too extensive. In *Escherichia coli*, DNA damage leads to induction of the SOS regulon, including UmuD and UmuC (UmuDC), which are thought to facilitate translesion synthesis on damaged DNA, with a concomitant decrease in replication fidelity (*2*, *3*). UmuDC and homologous proteins function only with their cognate operon partner (*4*) and require posttranslational processing to the mutagenically active UmuD′ (*2*, *3*). Umu-like proteins are found in enterobacteriaceae (*5*), although putative analogs have been identified in *Saccharomyces cerevisiae* (6) and *Caenorhabditis elegans* (*7*).

During the S phase in normal human cells, DNA replication produces ss DNA intermediates at least 100 kb long (*8*), probably resulting from displaced strands during asymmetric DNA synthesis (*8*). Other examples of non–fork-driven replication mechanisms are found in early *Xenopus* embryos, where perhaps as much as 30% of replication occurs on extensive regions of ss DNA (*9*), and in many viruses such as adenovirus.

Closed circular ss DNA is efficiently replicated to ds DNA in *Xenopus* oocytes, eggs (*10*), and nuclear extracts (*11*). Oocytes repair but do not replicate injected ds DNA, whereas *Xenopus* eggs and egg extracts replicate ~5% of injected ds DNA (*12*). We investigated how oocytes and nuclear extracts might replicate and repair ultraviolet (UV)–damaged ss DNA. One possibility is that UV damage might be bypassed completely and replicated at lower fidelity. However, the fidelity of ss to ds replication in oocytes (*13*) is as high as the fidelity of SV40 origin-dependent replication (*14*). Alternatively, DNA synthesis might be partially or completely blocked, or the damaged DNA might be degraded.

Single-stranded DNA was UV-irradiated (*13*, *15*) and injected into oocytes (Fig. 1A). Virtually no DNA synthesis was observed on UV-irradiated circular ss DNA containing as few as ~2 to 3 cyclobutane pyrimidine dimers (CPDs) per ss M13, whereas unirradiated ss DNA replicated fully. No partial replication products were observed, nor did replication occur on UV-irradiated linear ss M13 (*13*). UV-irradiated ss M13 recovered from oocytes yielded at least 5×10^3 fewer plaques than did unirradiated DNA after electroporation-mediated transformation. Photoreversal of UV lesions did not occur. The irradiated circular ss DNA was not degraded by the oocyte (Fig. 1).

To determine whether partial synthesis occurred, we measured the degradation rate of a labeled 1-kb complementary fragment annealed to ss M13 ± UV, injected into oocytes or added to extracts. The rate of degradation of this labeled fragment was too slow to have prevented detection of short-lived partial replication products (*13*). The 1-kb ds region on ss M13 was not further extended by the oocyte, which is consistent with observations that fork-driven replication does not occur in oocytes.

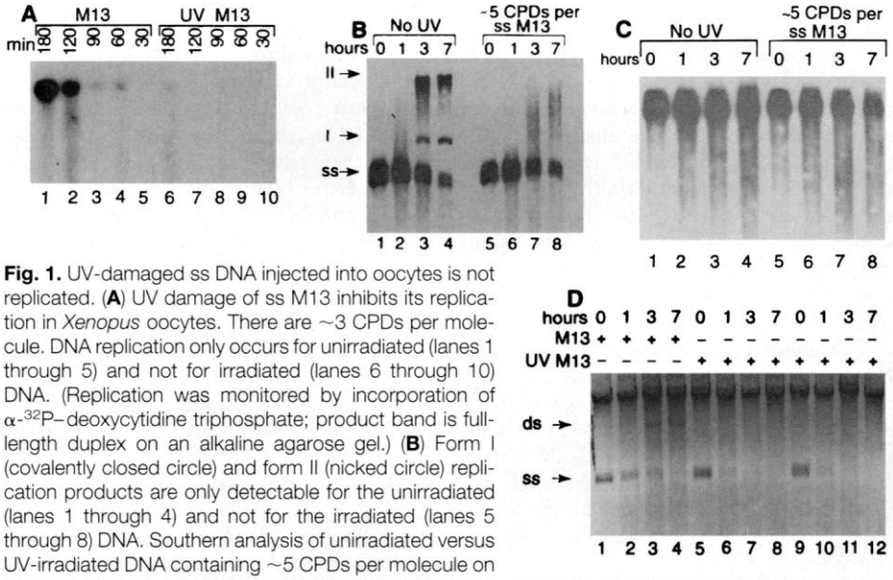

Fig. 1. UV-damaged ss DNA injected into oocytes is not replicated. (**A**) UV damage of ss M13 inhibits its replication in *Xenopus* oocytes. There are ~3 CPDs per molecule. DNA replication only occurs for unirradiated (lanes 1 through 5) and not for irradiated (lanes 6 through 10) DNA. (Replication was monitored by incorporation of α-^{32}P–deoxycytidine triphosphate; product band is full-length duplex on an alkaline agarose gel.) (**B**) Form I (covalently closed circle) and form II (nicked circle) replication products are only detectable for the unirradiated (lanes 1 through 4) and not for the irradiated (lanes 5 through 8) DNA. Southern analysis of unirradiated versus UV-irradiated DNA containing ~5 CPDs per molecule on a tris-acetate EDTA (TAE) gel and (**C**) on an alkaline gel. There is no degradation of unirradiated (lanes 1 through 4) or irradiated (lanes 5 through 8) DNA. Incubation times are indicated. (**D**) UV-irradiated ss M13 injected into oocytes is not replicated to form I and does not migrate as ss M13 tris-borate EDTA (TBE) gel. The ds replication product is only visible for the unirradiated (lanes 1 through 4) and not the irradiated (lanes 5 through 12) DNA. An EtBr-stained gel (reverse print) of recovered DNA is shown; there are ~3 CPDs per M13 molecule in lanes 5 through 8 and ~9 CPDs per M13 molecule in lanes 9 through 12. The dark band above the ds DNA is oocyte mitochondrial DNA. Injections and materials were as described (*11*, *15*, *25*), except that ss M13 was injected at 0.05, 0.2, and 2 mg/ml in (A), (B) and (C), and (D), respectively. The Southern hybridization probe was made from restriction fragment DNA with the Stratagene Prime-It II kit.

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Although no label was incorporated on ssDNA + UV, some of the irradiated ss M13 recovered from oocytes exhibited an altered electrophoretic mobility (Fig. 1, B through D), which is consistent with a protein or proteins binding damaged ss DNA. Although most of the unirradiated ss M13 replicated to forms I and II, irradiated ss M13 became undetectable. Southern (DNA) blots (Fig. 1, B through D) indicated that it was not degraded. Exhaustive protein removal restored the yield of UV–ss M13 to that of unreplicated ss M13 (*13*).

To determine whether the replication arrest functions in trans, we combined equal amounts of UV-irradiated single-strand bacteriophage ϕX174 with unirradiated M13 or vice versa. The unirradiated DNA replicated completely (Fig. 2A). Combination of irradiated with unirradiated ss M13 also resulted in replication of the unirradiated portion of M13 (*13*). Therefore, the oocyte replication arrest mechanism only applies to damaged template molecules.

Early *Xenopus* embryos replicate their genomes faster than does *E. coli* in log phase, whereas oocytes do not replicate their DNA at all. Oocytes are arrested at first meiotic prophase; matured oocytes are released from this arrest and proceed to metaphase of second meiotic division (*16*). When oocytes from the same batch were tested before and after progesterone-induced maturation, replication of irradiated ss M13 was seen only in the matured oocytes (Fig. 2B), which suggests the emergence in matured oocytes of a replicative bypass pathway or displacement of an antireplication protein or proteins. The replication arrest also existed in our oocyte nuclear extracts, which efficiently support DNA repair and ss-to-ds replication (*11*, *13*).

An error-prone replicative bypass system is found in *E. coli*, promoted by the mutagenesis proteins UmuD′, UmuC, and RecA (*17*). The Umu-like family of proteins is implicated in prokaryotic translesion DNA replication (*2*, *3*, *18*, *19*) and possibly in induced replisome reactivation (IRR) or replication restart (*18–20*). We therefore tested whether UmuD′C and their plasmid-derived analogs MucA′ and MucB (MucA′B) (*3*) might release replication arrest and facilitate bypass synthesis in oocytes injected with UV-irradiated ss M13 by injecting mRNA transcripts encoding the prokaryotic proteins. The mRNAs were first injected into the cytoplasm; the oocyte nuclei were later injected with label and ss M13 DNA. Combinations of either *mucA′* + *mucB* or *umuD′* + *umuC* facilitated replication of the irradiated ss M13 template (Fig. 3A). Messenger RNAs injected into the nucleus simultaneously with irradiated ss M13 also facilitated replication (Fig. 3B). Only cognate mRNA combinations promoted substantial replication. No replication occurred when control *EF1α* or non-cognate mRNA partners *mucA′* + *umuD′* or *umcC* + *mucB* were injected, and only limited synthesis was seen with *umuD′* + *mucB*.

How might UmuD′C and MucA′B proteins alleviate the replication arrest on damaged ss M13 DNA in oocytes? The bacterial proteins may directly interact with the *Xenopus* replication machinery or with a putative cis antireplication protein or proteins. The products synthesized from UV-irradiated ss M13 facilitated by UmuD′C or MucA′B proteins were more varied than those made from an undamaged template (Fig. 3B), which suggests that prokaryotic proteins might not allow extensive synthesis past CPDs in the oocyte. Alkaline agarose gel analysis of replication products on the damaged template (Fig. 3C) demonstrated that the prokaryotic proteins promoted initiation and elongation of DNA synthesis, but a substantial portion of the replication products were not full length. UmuD′C and MucA′B therefore abrogate the replication arrest in oocytes.

What *Xenopus* proteins are likely to interact with the bacterial mutagenesis proteins? Even with *E. coli* proteins, only activated RecA (RecA*) interacts with UmuD′ under limited conditions (*21*, *22*). In *Xenopus*, rapid cleavage of UmuD to UmuD′ occurs when UmuD is injected into either the oocyte nucleus or cytoplasm, and this cleavage in oocytes is abolished by serine protease inhibitors (*13*). Therefore, a modification required in *E. coli* also occurs via *Xenopus* proteins.

The crystal structure determination of UmuD′ revealed a likely DNA-mediated interaction between the NH_2-terminal region of UmuD′ and RecA* (*22*). An NH_2-terminal deletion mutant of UmuD′ that cannot promote mutagenesis in *E. coli* is also inactive in *Xenopus* (*13*). The mutant is nonmutagenic in *E. coli* because it requires an intact NH_2-terminal to interact with the RecA* filament, which suggests a similar interaction in *Xenopus*. At least three eukaryotic proteins—Rad51, Rad55, and Rad57—exhibit similar sequence homology to *E. coli* RecA (*23*). Purified *Xenopus* Rad51 (*24*) does not yield an interaction analogous to ss DNA–UmuD′–RecA gel shifts (*13*). The inability of *Xenopus* Rad51 to gel-shift UmuD′ is consistent with the notion that there may be a family of RecA-like molecules in eukaryotes, rather than one such as in *E. coli*, and implies

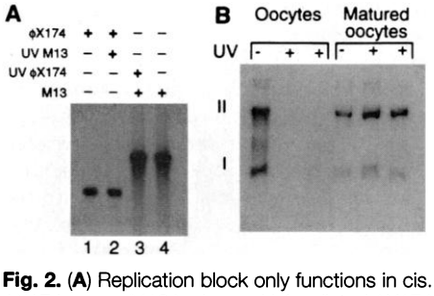

Fig. 2. (**A**) Replication block only functions in cis. ϕX174 versus M13 is shown. Unirradiated ϕX174 (lanes 1 and 2) or M13 (lanes 3 and 4) is replicated in the presence or absence of UV-irradiated M13 or ϕX174, respectively. Methods were as in Fig. 1, except that TBE gel was used. Recovered DNA was linearized with Pst I. (**B**) Progesterone-matured oocytes allow replication of UV-irradiated ss M13 (~5 CPDs per molecule, duplicate samples). Progesterone-matured oocytes were injected in 1× Ringer solution with 5% Ficoll to avoid activation and promote healing (*26*).

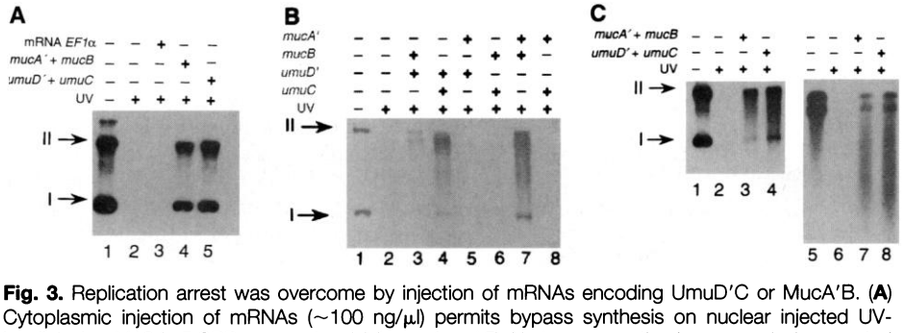

Fig. 3. Replication arrest was overcome by injection of mRNAs encoding UmuD′C or MucA′B. (**A**) Cytoplasmic injection of mRNAs (~100 ng/μl) permits bypass synthesis on nuclear injected UV-irradiated ss M13 (~5 CPDs per molecule) (lanes 4 and 5). No bypass synthesis occurred when control *EF1α* mRNA (lane 3) was injected (~100 ng/μl) or no mRNA was injected (lane 2). Cytoplasmic injections were done ~2 to 3 hours before nuclear injection of ss M13 and label. (**B**) Only cognate combinations of mRNAs for Umu or Muc proteins (lanes 4 and 7) permit bypass replication on UV-irradiated ss M13 (~3 CPDs per ss M13). Single-stranded M13, label, and mRNAs were all injected simultaneously into the nucleus. (**C**) TBE (lanes 1 through 4) and alkaline (lanes 5 through 8) gel analysis of replication products detected after injection of mRNAs for UmuD′C and MucA′B. Messenger RNA transcripts from *umu* or *muc* genes under T7 RNA polymerase promoters (*21*) were prepared with Ambion's (Austin, Texas) in vitro transcription kit or by equivalent methods. Messenger RNA integrity and concentration were determined by 1.5% agarose gels. Injected UmuD′ mRNA yields a protein detectable by protein immunoblot analysis (*13*).

complex interactions between *Xenopus* proteins and the bacterial mutagenesis proteins to abrogate replication arrest.

Although entering apoptosis might be appropriate for cells with heavily damaged genomes, there may be a local checkpoint mechanism in vertebrates that permits replication past small numbers of unrepairable lesions. Our results suggest that some eukaryotic cells contain a protein or proteins that prevent replication on damaged ss DNA and that other cells might possess a bypass system related to that found in prokaryotes.

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27. We thank J. K. Saxena, who participated in the early injection experiments with irradiated ss M13; J. Hauser for constructing the T7 mucA′ and mucB plasmids; V. Doseeva for testing replication of UV-damaged ss M13 with multiple oocyte nuclear extracts and transformation of DNA recovered from injected oocytes into competent cells; T. Horii for providing a *Xenopus RAD51* cDNA clone; Y. Shiloach and the NIDDK Biotechnology Unit for large-scale bacterial preparations; R. S. Lloyd for providing T4 UV endonuclease; and T. Kunkel, N. Nossal, and S. H. Wilson for comments on the manuscript.

8 March 1996; accepted 25 March 1996

Thymine-Thymine Dimer Bypass by Yeast DNA Polymerase ζ

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The *REV3* and *REV7* genes of the yeast *Saccharomyces cerevisiae* are required for DNA damage–induced mutagenesis. The Rev3 and Rev7 proteins were shown to form a complex with DNA polymerase activity. This polymerase replicated past a thymine-thymine *cis-syn* cyclobutane dimer, a lesion that normally severely inhibits replication, with an efficiency of ~10 percent. In contrast, bypass replication efficiency with yeast DNA polymerase α was no more than 1 percent. The Rev3-Rev7 complex is the sixth eukaryotic DNA polymerase to be described, and is therefore called DNA polymerase ζ.

Mutations generated by spontaneous or mutagen-induced damage to DNA are thought to be a major contributing cause of many cancers. Many of these mutations probably arise during lesion bypass, that is, during replication past the site of DNA template damage. Bypass replication requires different protein products from those used in the replication of undamaged DNA, but the process is poorly understood. Translesion synthesis in *Escherichia coli* is usually carried out by DNA polymerase (Pol) III, the major chromosomal replicase, together with at least three accessory proteins, the products of the *recA*, *umuC*, and *umuD* genes (*1*). The Umu proteins form a complex, consisting of a dimer of UmuD′ (the proteolytically processed product of UmuD) and a monomer of UmuC, which RecA may target to the site of the DNA damage. The bound Umu-RecA complex appears to enhance the processivity of DNA Pol III, thus relieving the severe inhibition to elongation imposed by the lesion.

In the budding yeast *S. cerevisiae*, at least three genes are required for DNA damage–induced mutagenesis. The *REV1* gene (*2*) encodes a protein that has weak homology with the *E. coli* umuC protein; the *REV3* gene encodes a protein with sequence motifs characteristic of a DNA polymerase (*3*), although no activity attributable to this gene has previously been detected in cell extracts; and the *REV7* gene encodes a protein with no sequence similarities to any other protein in the National Center for Biotechnology Information sequence database (*4*). None of these genes is essential for viability, and each has a very similar mutant phenotype: *rev* deletion strains exhibit little induced mutagenesis and are slightly more sensitive than the wild type to DNA-damaging agents, but their repair and recombination functions are otherwise normal. This phenotype suggests that the proteins encoded by *REV* genes are dedicated to bypass replication.

To investigate the properties of the putative Rev3 DNA polymerase, we purified a glutathione S-transferase–Rev3 fusion protein (Gst-Rev3p), overexpressed in yeast, by affinity chromatography on glutathione-Sepharose. SDS–polyacrylamide gel electrophoresis (PAGE) analysis of the fraction obtained from this one-step purification showed a single major band of protein of ~205 kD, as expected for the Gst-Rev3 fusion (Fig. 1). Gst-Rev3p consistently showed DNA polymerase activity that was 10 to 100 times that of control preparations of Gstp alone, but this activity was unstable and difficult to study. We next investigated whether Rev3p exists as part of a multiprotein complex, which might explain its instability. We used the yeast two-hybrid system (*5*) to test for interactions between Rev1p, Rev3p, and Rev7p and to look for other proteins that might interact with the Rev proteins (*6*). A weak positive signal for interaction was observed when the LexA-Rev3 and Gal4-Rev7 fusions were paired, which suggested that Rev3p interacts with Rev7p. Compelling evidence for this interaction was obtained when the LexA-Rev3 fusion was used to screen a Gal4 fusion library of yeast cDNA for potential interacting proteins. Only two positive clones were identified in a screen of 10^6 transformants; both were in-frame fusions to Rev7, one fused at amino acid 8 and the other at amino acid 10. These results indicate that

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