Xenopus egg lysates repair heat-generated DNA nicks with an average patch size of 36 nucleotides

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ABSTRACT

Base excision repair (BER) is an essential DNA repair pathway since it processes spontaneous (endogenous) DNA damage such as abasic sites, oxidized and alkylated bases, as well as mismatches arising from deamination of cytosine and 5-methylcytosine. Some of these lesions are repaired by the exchange of a single deoxynucleotide [Dianov, G. *et al.* (1992) *Mol. Cell. Biol.* 12, 1605–1612; Wiebauer, K. and Jiricny, J. (1990) *Proc. Natl. Acad. Sci. USA*, 87, 5842–5845] or a few deoxynucleotides [Matsumoto, Y. *et al.* (1994) *Mol. Cell. Biol.*, 14 6187–6197]. Here we report that DNA single strand breaks induced by hyperthermic conditions are repaired with an average patch size of ~36 nt in *Xenopus laevis* egg lysates.

INTRODUCTION

Since the establishment of the first *in vitro* repair system by Wood *et al.* (4), cell-free lysates have become potent tools to study molecular mechanisms of DNA repair. A major advantage of cell-free systems is that highly purified plasmid DNA with defined lesions can be introduced into repair reactions together with purified repair components or specific antibodies. However, during storage of plasmids nicks accumulate. This spontaneous nicking may confound the interpretation of repair studies. In the present report, we investigated the re-joining of spontaneous nicks. The results show that long patches are inserted. Thus, only a few spontaneous nicks in addition to the defined lesions in repair assays may seriously affect the analysis of repair patch sizes and repair efficiency.

METHODS

Preparation of *Xenopus laevis* egg lysates

Lysates were prepared by the method of Almouzni and Méchali (5) with some modifications. Unfertilized eggs were obtained from female frogs after subcutaneous injection with 250–300 U of human chorionic gonadotropin (Sigma). Six to eight hours after the first injection, frogs were injected again with 750–800 U and placed in separate siliconized tanks containing high-salt

Barth's saline (HSBS; 15 mM Tris–HCl, pH 7.6, 110 mM NaCl, 2 mM KCl, 1 mM MgSO$_4$, 0.5 mM Na$_2$HPO$_4$, 2 mM NaHCO$_3$). Eighteen hours after the first injection, extruded eggs were carefully collected, rinsed in HSBS, and allowed to swell in re-distilled water. Eggs were dejellied in 0.1 × HSBS containing 2% cysteine. After three washes with ice-cold extraction buffer [20 mM Hepes, pH 7.5, 70 mM KCl, 1 mM DTT, 5% (w/v) sucrose], eggs were centrifuged for 30 min at 12 000 g, 4°C. The ooplasmic fraction was recovered and subjected to a second centrifugation for 60 min at 150 000 g, 4°C. The clear cytoplasmic fraction was collected, and 100 µl aliquots were stored at −80°C. The extracts contained endogenous cytoplasmic components diluted ~2-fold, including 50 µM dNTPs (6).

In vitro repair reactions

Standard 20 µl repair reaction mixtures contained 175 µg whole cell extract protein (10 µl egg lysate) and were supplemented with 35 mM KCl, 7.5 mM MgCl$_2$, 100 µM NAD$^+$, 2 mM ATP, 20 µM each of dATP, dCTP and dTTP, 8 µM dGTP, 2 µCi of [α-^{32}P]dGTP (3030 Ci/mmol; 7420 d.p.m./pmol in reaction mixture), 40 mM phosphocreatine, 1 µg creatine phosphokinase and 3% glycerol. Stock solutions of the individual assay components were prepared in buffer C and stored at −80°C, with the exception of creatine phosphokinase which was freshly prepared. Reactions were initiated by the addition of nicked plasmid DNA and incubated at 30°C. Repair reactions were stopped with 40 mM EDTA, pH 8, and incubated for 5 min at 37°C in the presence of RNase A (80 µg/ml). SDS was added to 0.5%, and proteinase K to 190 µg/ml. Samples were incubated for 30 min at 37°C, followed by extraction with phenol–chloroform. Plasmid DNA was precipitated, washed, and electrophoresed on 1% agarose gels containing 0.5 µg/ml ethidium bromide. Band intensities were quantified in the photographic negative and the autoradiogram of the dried agarose gel by scanning densitometry (Image Quant software). To calibrate densitometry results, bands were excised from the gel and analyzed by Cerenkov counting.

Nicked plasmids

Standard procedures were used to propagate and purify plasmid pBS (Bluescript II SK+/– phagemid, 2961 bp, Stratagene) in *E.coli* DH5α. Purification of supercoiled forms occurred by centrifugation in ethidium bromide–CsCl and sucrose gradients

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(7). To create single-stranded DNA breaks 10 μg of plasmid DNA was incubated for 7, 12 or 31 h in 50 μl TE, pH 8, at 56°C.

RESULTS

Re-joining of single stranded DNA breaks

Due to the chemical instability of the DNA (8), nicks arise upon storage. This spontaneous process can be accelerated under hyperthermic conditions. Figure 1a shows that DNA was increasingly nicked by incubation at 56°C as a function of time. Initially, already 6% of the plasmids were nicked. After 7, 21 and 31 h of incubation, 28, 43 and 47% of the plasmids were nicked, respectively. Poisson statistics revealed that each plasmid contained 0.06, 0.33, 0.56 and 0.64 nicks, for 0–31 h of incubation. After 30 min repair, nicks were clearly resealed (Fig. 1b) and essentially all plasmids were once again supercoiled. The small fraction of plasmids in the nicked position is caused manually during the experiment. Figure 1c and d shows a positive correlation between the number of nicks and the incorporation of radionucleotides. Quantitative analyses (Fig. 1e) revealed an average patch size of 36 ± 2.9 (mean ± S.D.) bases per nick repaired.

DISCUSSION

A prerequisite for the quantification of repair efficiencies is to determine the number of damages per plasmid and the repair patch size. In this report we show that spontaneous DNA nicks are rapidly repaired in *Xenopus* egg lysates. 100% of plasmids containing an average of 0.64 nicks (31 h heat treatment) were re-joined within 30 min, indicating complete repair. Since nicked plasmids contain unligatable ends (data not shown), at least three specific enzymes are required for repair: an exonuclease or phosphatase to remove various terminal groups preventing DNA synthesis or ligation, a DNA polymerase for gap filling, and a DNA ligase. Theoretically, it would be sufficient to replace one or a few nucleotides, but an average of 36 bases was excised, indicative of a long patch repair mechanism. This finding is important when the subject of investigation is a short patch size base excision repair mechanism. Even after purification of closed circular plasmids on sucrose gradients a small fraction of plasmids is nicked. These nicks are obviously not repaired by short patch BER mechanisms and contribute significantly to the observed repair signal.
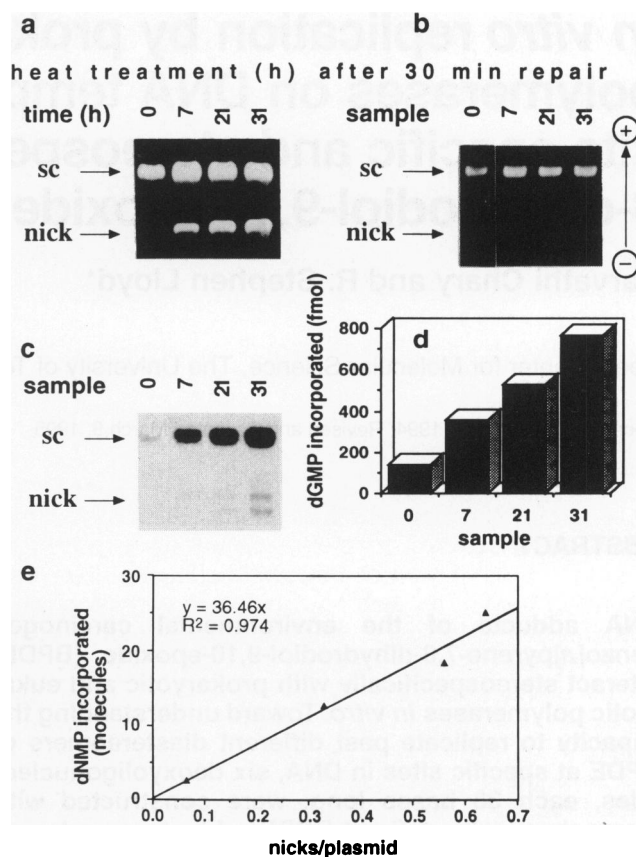
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Figure 1. Repair of DNA single strand breaks by *Xenopus* egg lysates. (**a**) 250 ng of pBS DNA incubated for the indicated times at 56°C were electrophoresed on a 1% agarose gel containing ethidium bromide to monitor nicking of the plasmids as a function of time. (**b**) 250 ng of plasmids were incubated under repair conditions as described in methods for 30 min, and the recovered DNA was separated on a 1% agarose gel in the presence of ethidium bromide. (**c**) Autoradiogram of the dried agarose gel, (**d**) scanning densitometric analysis of the autoradiogram, and (**e**) determination of the repair patch size from the slope of deoxynucleotide monophosphate incorporation versus the nick frequency. sc and nick indicate the positions of supercoiled and nicked pBS, and the direction of migration is indicated by an arrow.

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