

The repair patch of *E.coli* (A)BC excinuclease

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ABSTRACT

The size of repair patch made by *E. coli* DNA polymerase I (PolI) following the removal of a thymine-psoralen monoadduct by *E. coli* (A)BC excinuclease was determined by using an M13mp19 DNA with a single psoralen monoadduct at the polylinker region. Incubation of this substrate with (A)BC excinuclease, PolI and a combination of 3 dNTP plus 1 dNTP(α S) for each nucleotide, and DNA ligase resulted in a repair patch with phosphorothioate linkages. The preferential hydrolysis of phosphorothioate bonds by heating in iodoethanol revealed a patch size-with minimal nick translation-equal in length to the 12 nucleotide gap generated by this excision nuclease.

INTRODUCTION

In *Escherichia coli* nucleotide excision repair is initiated by (A)BC excinuclease which hydrolyzes the 8th phosphodiester bond 5' and the 4th or 5th phosphodiester bond 3' to pyrimidine dimers and other nucleotide mono- and diadducts (1,2). Thus, if the excision gap is filled in without nick translation a 12–13 nucleotide-long patch should be produced. The patch size has been determined by several methods *in vivo*. Cooper and Hanawalt (3) estimated a patch size of 20–30 nucleotides from the density shift of DNA fragments containing repair patches with BrdU instead of thymine. Ley and Setlow (4) obtained an average patch size of 16–24 nucleotides while Rothman (5,6) and Carlson and Smith (7) obtained 10–30 nucleotides by the BrdU photolysis method. Ben-Ishai and Sharon (8) measured the number of nucleotides incorporated per pyrimidine dimer released in a permeabilized cell system and found a value of 13–16 nucleotides per repair patch.

Two *in vitro* studies have been conducted on the patch size of (A)BC excinuclease. Caron *et al.* (9) used UV-irradiated pBR322 which contained an average of one pyrimidine dimer per molecule and incubated DNA with UvrA, B, and C in the absence of PolI to obtain the number of nicks made by the enzyme, and in the presence of PolI + helicase II + ligase and [α -³²P]dNTPs to measure repair synthesis, and reported 12.6 nucleotides incorporated per nick. Van Houten *et al.* (10) used M13mp19 containing a single psoralen adduct at a unique site which was treated with (A)BC excinuclease along with DNA

polymerase I, helicase II, and ligase and [α -³²P]dNTP. The site of incorporation of label was examined by restriction analysis and it was found that no label was incorporated beyond 5 nucleotides 5' to the 5' incision site and only 17% of the label was incorporated into the 33 bp beyond the 3' incision site; no detectable incorporation was found beyond that. Thus, both *in vivo* and *in vitro* studies indicate that the (A)BC excinuclease generated gap is filled in and ligated without significant nick translation. However, none of the studies mentioned had the resolution or precision required to rule out some gap enlargement in 5' direction or a few nucleotide nick translation in 3' direction.

In the present study we have used a defined substrate and a new method to determine the patch sizes after repair. We find that the repair patch for (A)BC excinuclease is 12 nucleotides, with only marginal nick translation.

MATERIALS AND METHODS

Materials

The UvrA, UvrB and UvrC subunits of (A)BC excinuclease were purified as described previously (11). DNA polymerase I (PolI), T4 polynucleotide kinase, T4 DNA ligase and restriction endonucleases were purchased from Boehringer-Mannheim. [γ -³²P]dNTP was from DuPont-New England Nuclear. All four dNTP(α S) compounds were obtained from Amersham.

Methods

M13mp19 with a 4'-hydroxyl-4,5',8-trimethylpsoralen (which will be simply referred to as psoralen)-thymine monoadduct at the center of the KpnI site in the polylinker region was prepared by the method of Kodadek and Gamper (12). Following synthesis covalently closed duplex DNA was purified by CsCl-ethidium bromide density gradient centrifugation. The repair synthesis buffer contained 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM MgCl₂, 2 mM ATP, 5 mM dithiothreitol, and 3 dNTPs plus 1 dNTP(α S) at 40 μ M each plus 2 units of PolI and 2 units of T4 DNA ligase, and bovine serum albumin 50 μ g/ml.

The repair patch assay for (A)BC excinuclease was conducted as follows. The reaction mixture contained in 50 μ l repair synthesis buffer ~0.1 nM M13mp19(Pso), 10 nM UvrA, 200 nM UvrB, 30 nM UvrC, 3 dNTPs and 1 dNTP(α S) each at 40 μ M. The reaction mixtures were incubated at 37°C for 30 min.

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the size and sequence of the repair patch of (A)BC excinuclease (Figure 1). Circular DNA containing a single adduct at a unique site is incubated with a mixture of (A)BC excinuclease, DNA polymerase I, DNA ligase, ATP, 1 dNTP(α S) and 3 dNTPs. Then the DNA is extracted, digested with a restriction enzyme which cuts at a reasonable distance from the adduct and the fragment carrying the adduct (or the repair patch) is kinased, treated with iodoethanol and separated on a sequencing gel alongside the sequence of the damaged strand. It is expected that there will be a sequence ladder in repaired DNA only in the region of the repair patch.

The Repair Patch of (A)BC Excinuclease

The M13mp19 replicative form containing a psoralen furan side-thymine monoadduct at the unique KpnI site was used as a substrate to measure the patch size of the excision nuclease by the phosphorothioate method. Four repair reactions were carried out with 3 dNTPs and 1 dNTP(α S) using a different dNTP(α S) in each reaction. Following repair synthesis the DNA was digested with PvuII which cuts 102 bp 5' and 220 bp 3' to the modified thymine. The resulting 322 bp fragment was terminally labeled and then the DNA was heated in 7% iodoethanol at 95°C for 3 min and analyzed on sequencing gels. Iodoethanol ethylates the phosphorothioate groups and makes them susceptible to hydrolysis at high temperature but has no effect on the phosphodiester bonds. Thus, heating the repaired DNA results in cleavage at the sites of incorporation of dNTP(α S), giving rise to a sequence ladder only at the region of the repair patch. The results are shown in Figure 2. Note that because of the nature of the chemical reaction, fragments of both 3'-hydroxyl and 3'-phosphate termini are produced (13) and as a consequence each base is represented by two-closely spaced bands. With this consideration in mind, then, the sequence of the repair patch in Figure 2 is unambiguous. It appears that PolI simply fills in the 12 nucleotide gap thus generating a repair patch of this size in the vast majority of molecules. It is clear that there is no trimming 5' to the 5' incision site of the (A)BC excinuclease. This result has been obtained at a variety of DNA polymerase and ligase conditions and therefore we believe these results reflect the *in vivo* conditions. However, under these *in vitro* conditions a 2-nucleotide nick translation by PolI is observed in about 50% of the molecules (compare band intensities of the G immediately 5' to the modified T and of the G band below the 3' incision band in the G lane of the phosphorothioate sequence) and as much as 10 nucleotide nick translation is seen in a much smaller fraction of DNA. Whether very long patches (> 1000 nucleotides) are ever produced at very low frequency as has been seen *in vivo* (3) cannot be ascertained from our assay. Also of interest is the fact that the presence or absence of helicase II did not affect the size of the repair patch (data not shown).

In conclusion, we have described one assay to directly visualize the repair patch of *E. coli* (A)BC excinuclease. The results obtained with this assay indicates that the patch size is 12 nucleotides, and there is only minimal nick translation in filling in the single stranded DNA 'gaps' of this excision repair pathway. The results, by nature of the *in vitro* systems used, are more precise than the values obtained *in vivo* but in general are in excellent agreement with some of the more precise *in vivo* patch values. Furthermore, this assay is not restricted to (A)BC excinuclease and can be adapted to determine the sizes of repair patches made during nucleotide excision repair in other systems as well as for determining the exact size of the repair patch in

base excision repair, provided circular DNAs with a uniquely located adducts are available.

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