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Replication of M13 single-stranded DNA bearing a site-specific ethenocytosine lesion by *Escherichia coil* cell extracts

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ABSTRACT

Previous investigation on the mutagenic effects of 3, N^4-Ethenocytosine (εC), a nonpairing DNA lesion, revealed the existence of a novel SOS-independent inducible mutagenic mechanism in *E. coli* termed UVM for UV modulation of mutagenesis. To investigate whether UVM is mediated by an alteration of DNA replication, we have set up an *in vitro* replication system in which phage M13 viral single-stranded DNA bearing a single site-specific (εC) residue is replicated by soluble protein extracts from *E. coli* cells. Replication products were analyzed by agarose gel electrophoresis and the frequency of translesion synthesis was determined by restriction endonuclease analyses. Our data indicate that DNA replication is strongly inhibited by εC, but that translesion DNA synthesis does occur in about 14% of the replicated DNA molecules. These results are very similar to those observed previously *in vivo*, and suggest that this experimental system may be suitable for evaluating alterations in DNA replication in UVM-induced cells.

Key words: *Ethenocytosine, M13, in vitro replication, cell extract.*

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INTRODUCTION

Mutagens usually react with DNA to form DNA adducts (lesions), which, if unrepaired, can lead to mutations. Thus, the assessment on the biological consequences of major classes of DNA lesions is useful for understanding the mechanism of carcinogenesis. Traditionally, mutagenic DNA lesions have been classified into two broad categories. Mispairing lesions, such as O^6-methylguanine and DNA uracil, generally do not stop replication because they can pair with an incoming base, even if the pairing is 'wrong' in a biological sense. Therefore, mispairing lesions possess high mutagenic efficiency without any requirement for cellular functions other than those required for normal DNA replication. On the other hand, noninstructive lesions, such as abasic sites and bulky DNA lesions, lack accessible template information, so that DNA replication is blocked when DNA polymerase encounters the lesions. In the well-studied Escherichia coli model, the survival and mutagenic effects of noninstructive lesions depends on induced functions such as those provided by the SOS system[I-3]. Three SOS inducible proteins, namely, RecA, UmuD' and UmuC are believed to facilitate translesion DNA synthesis by a mechanism that is not understood[4, 5]. The result of this process is increased survival at the cost of increased mutagenesis.

3, N^4-ethenocytosine (εC) is a highly mutagenic exocyclic DNA lesion induced by a number of industrial carcinogens such as vinyl chloride[6] and ethyl carbonate[7] as well as by certain endogenous agents[8]. Because of the etheno bridging of two of the three hydrogen-bonding positions (Fig 1), εC is expected to have the template characteristics of a noninstructive lesion. Recent studies on the mutagenic properties of the εC lesion, including those involving the transfection of gapped duplex M13 DNA[9-11] or of single-stranded M13 viral DNA (ssDNA[12, 13]), and those involving primer elongation in vitro by *E. coli* DNA polymerase I[14, 15] are consistent with the possibility that εC is noninstructive. However, εC did differ from other common examples of noninstructive / replication-arresting lesions in that mutagenesis at εC did not require a functional *recA* gene, a crucial regulatory and enzymatic element of SOS system[10]. Further investigation revealed the existence of a novel mutagenic phenomenon termed UVM for *UV* modulation of mutagenesis[13-16]. UVM appears to represent a SOS independent DNA damage-inducible cellular response to genotoxicants[17, 18].

To investigate whether UVM is mediated by a transient alteration of DNA replication, we have sought to develop an *in vitro* system for a high-resolution analysis of translesion DNA synthesis by *E. coli* cell extracts. Because replication of the filamentous bacteriophage M13119, 20] is well-understood, we have based our system on the conversion of M13 ssDNA to double-stranded replicative form (ds; RF) DNA. In the first stage of M13 ssDNA replication, DNA synthesis is initiated at a unique site called the minus-strand origin. A 20-nucleotide RNA primer[21] is synthesized at the site by the host RNA polymerase, and the primer is subsequently elongated by DNA

polymerase III holoenzyme. After DNA synthesis around the ssDNA circle, DNA polymerase I is believed to excise the primer RNA and to refill the gap with DNA. Subsequent actions of DNA ligase and DNA gyrase generate a covalently closed supercoiled DNA molecule (form I or RF-I]. Here we describe the establishment of an in vitro system for replicating M13 ssDNA containing site-specific lesion.

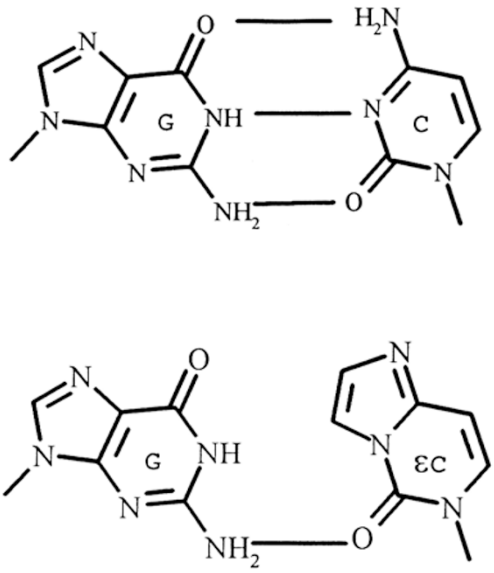

Fig 1. Structure of 3, N^4-ethenocytosine. (Top) A normal G:C pair with three Watson-Crick hydrogen bonds. (Bottom) A G:εC "pair" showing the effect of etheno bridging on base pairing.

MATERIALS AND METHODS

Materials

Sources are as follows: unlabeled deoxynucleoside triphosphates and ribonucleoside triphosphates, Pharmacia; [α-^{32}p]dCTP (10μCi/μl, 6000Ci/mmol), Amersham; All the enzymes including restriction endonucleases, T4 DNA ligase, Proteinase K, New England Biolabs; DNA purification kit, QIAGEN; other chemicals, Sigma. Oligonucleotides were synthesized by New Jersey Medical School Molecular Resource Facility and were purified by high-resolution gel electrophoresis before use.

Bacterial and phage strains

E. coil K12 strains KH2 Δ*(lac-pro) trpE4777* (F' *lacI*-qZΔM15 pro$^+$); KH2R Δ(*srlR-recA*) 306::Tn10(Tet^r) in KH2 and KH3 recA-56 Δ (*lac-pro*) *trpE4777*F- have been described[22]. M13mp7L2 is a derivative of phage M13mp7[23] (Fig 2A).

Construction of M13 single-stranded DNA bearing a site-specific εC

Procedures used for the construction of M13 ssDNA bearing a site-specific εC residue (εC-DNA) or the corresponding control construct (C-DNA) bearing normal cytosine in the place of the εC residue have been described previously[13] and are summarized in Fig 2. The DNA constructs prepared by the above procedures were subjected to further treatment as follows. The ligation mix (molecule 3 in Fig 2) was deproteinized by a phenol extraction, and the DNA was recovered by ethanol precipitation attd resuspension in a small volume of water. To completely remove the 57-mer scaffold, the DNA was loaded on a QIAGEN (tip 20) resin column. The oligonucleotides were washed out of the column with 1.0 M NaCl, pH 7.0 (Buffer QC), and ssDNA was eluted with 1.25 *M* NaCl, pH 8.5 (Buffer QF).

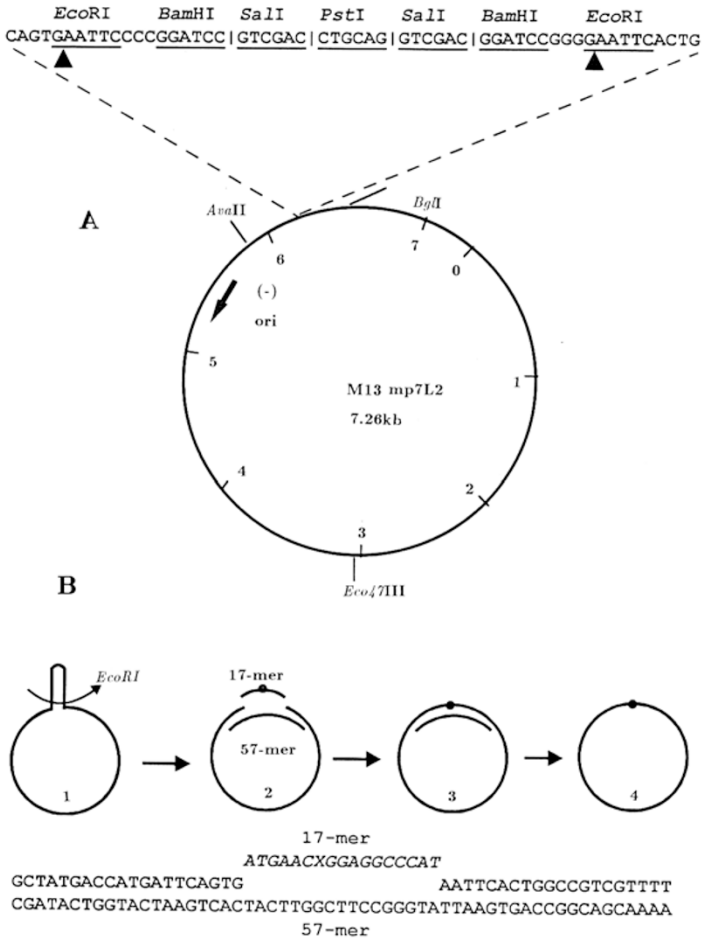

Fig 2. Map of M13mp7L2 DNA and construction of M13 ssDNA bearing a site-specific ethenocytosine lesion.

(A) M13mp7L2 was derived from M13mp7 by replacing a dinucleotide (5'-ACGAATTC-) at the 5' boundary of the mp7 polylinker sequence with a tetranucleotide (5'-*CAGT*GAATTC-). The polylinker sequence of the mp7L2 ssDNA forms an extended hairpin structure that is expected to be more readily cleaved by *EcoRI* (See Panel B, molecule 1). The location of the origin for minus-strand synthesis, and relevant restriction sites in the double-stranded (ds) RF DNA are shown. Digesting RF DNA with AvaII and Eco47III will give rise to two fragments with the length of 4.38 and 2.88 kb.

(B) Construction of εC-containing ssDNA involves the following steps: (1) linearization of M13 ssDNA by EcoRI digestion; (2) annealing with the 57-mer scaffold and a 17-mer containing the εC residue (the relevant sequences are shown at the bottom, with X represents the lesion residue); (3) ligation and (4) removal of 5T-mer scaffold. Identical procedures are used for the construction of the control construct (C-DNA) in which normal cytosine replaces εC in the 17-mer. Experimental procedures have been described in detail previously. Note that during the construction the polylinker sequence is removed and replaced by a 17-mer bearing the εC residue. DNA constructs used as templates for *in vitro* DNA synthesis were subjected to further purification as described in the text.

Preparation of E.coli cell extra

E. coli cell extract (fraction II) was prepared by a method adapted from Fuller et a1[24] and Khan et a1[25] as summarized below. *E. coli* cells were grown in 1300 ml LB medium at 37℃ to late-log phase ($OD_{600} \approx 1.0$) and harvested by centrifugation (1500 × g for 10 min at 4℃). Cell pellets were rinsed with a small volume of buffer A (25 m*M* Hepes, pH 7.6 / 1 m*M* EDTA), resuspended in 6 ml buffer A and frozen in a dry ice-ethanol bath before storage at -70℃. Frozen cell suspensions were thawed at 15℃ and adjusted to 80 m*M* KCl, 2 mM dithiothreitol, and 200 μg/ml egg lysozyme. After incubation at 0℃ for 20 min, the cells were lysed by two rounds of freezing (-70℃ for 2 min) and thawing (15℃ for 20 min). Cell lysates were clarified by centrifugation at 100,000 × g for 30 min at 4℃ (fraction I). To remove chromosomal DNA, 0.1 volume of 30% (w/v in water) streptomycin sulfate was added to fraction I followed by stirring at 0℃ for 30 min. The resulting DNA precipitates were removed by centrifugation at 20,000 × g for l0 min at 4℃. To each 1 ml of the supernatant, 0.28 g (for 45% saturation) or 0.47 g (for 70 % saturation) of ammonium sulfate was added slowly with stirring over a 15 min period at 0℃, and the suspension was stirred for an additional 20 min. Protein precipitate was collected by centrifugation at 20,000 × g for 10 min at 4℃ The pellets were resuspended in 0.5 ml of buffer B (25 m*M* Hepes, pH 8.0, 0.1 mM EDTA, 2 m*M* DTT), and dialyzed against a 100-fold volume of buffer B at 0℃ for 2 h. The dialysate (fraction II) was distributed as 50 μl aliquots, frozen in dry ice-ethanol bath, and stored at -70 ℃. Protein concentration of the cell extract (fraction II) was in the range of 40-80 mg/ml as determined by Bio-rad protein assay using bovine serum albumin as a standard.

In vitro replication assay

The typical reaction mixture (70 μl) contained 40 m*M* Tris-HCl (pH 8.0), 100 m*M* KCl, 12 m*M* magnesium acetate, I m*M* dithiothreitol, 5% ethylene glycol (v/v), 2 m*M* ATP, 0.5 m*M* each of UTP, GTP and CTP, 50 μ*M* each of NAD, cAMP, dATP, dGTP and dTTP, 20μ*M* of dCTP, 2μl $\alpha[^{32}p]$dCTP (10 μCi/μl, 6000 Ci/mmol), 200 ng of ssDNA, and 1 mg (protein) of E. coli cell extract. The reaction mix was incubated at 32 ℃ for the indicated time. The contents were deproteinized by incubation with proteinase K at concentration of 0.4 mg/ml for 30 min at 37 ℃ followed by phenol extraction. Aliquots of replication products at this stage were either directly analyzed by gel electrophoresis on 1% agarose in TAE (40 m*M* Tris-acetate, 1 mM EDTA, pH 8) buffer containing 0.5μg/ml ethidium bromide, or were subjected to ethanol precipitation and subsequent restriction enzyme digestion before analysis as above. The positions of the RF-I (supercoiled circular) and RF-II (nicked circular) DNA were identified with the help of authentic markers. Replication products were visualized by autoradiography of the dried gel, and quantitated by computing densitometry as described[14].

RESULTS

Optimization of in vitro replication system

Several procedures for preparing cell-free protein extracts that can support the replication of different types of DNA templates have been described[24-26]. We essentially followed the method of Fuller et al. [24], that is suitable for supporting DNA replication initiated at *oriC*, the origin of *E. coli* chromosome. Fuller et al.[24] had shown that maximal replicating activity was present in proteins precipitated from cell extract (fraction I) with 45% saturated ammonium sulfate. Investigators who studied replication of single-stranded plasmid DNA *in vitro* instead collected proteins precipitated with 70% saturated ammonium sulfate[25, 27]. In preparing cell extract from *E. coil* KH3, we tested the both conditions using the modified protocol

described in Materials and Methods. We did not observe a significant difierence in replicating activity between the two procedures(data not shown). Considering that nnlch more proteins were recovered by 70% saturated ammonium sulfate fractionation, we selected this condition in the succeeding experiments.

Fig 3 shows a time course of *in vitro* replication of M13mp7L2 ssDNA (used as a control or reference DNA) in the presence of *E. coli* KH3 cell extract. [α^{32}P]-dCTP was used in the replication reaction so that only newly synthesized strands can be seen from autoradiograph. At 15 and 30 min, considerable amount of DNA synthesis was observed, but most of the DNA was RF-II (nicked or gapped circular). Supercoiled circular DNA (RF-I) appeared at 45 min, with optimal DNA synthesis occurring at 90 min at which time RF-I constituted about 40%of the products. After 120 min, the amount of DNA started to decrease, presumably due to degradation by nuclease activities present in the extract. Although the level of RF-1 was about 40% in our replication system using M13 ssDNA (7.3 KB)as the template, we obtained RF-I DNA at about 60%when replicating the smaller ssDNA genome (2.6 KB)of pLitmus 28, a phagemid containing M13 origin of replication(data not shownl). It is not clear whether the lack of complete conversion of RF-II to RF-I indicates limiting amounts of the responsible enzymes, or represents the equilibrium between synthetic and degradative activities present in the extract.

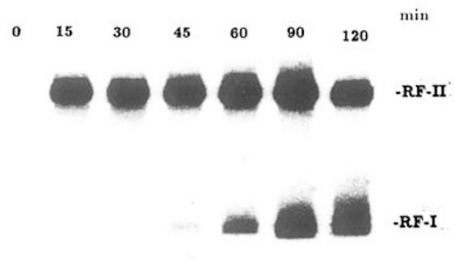

Fig 3. Characteristics of the *in vitro* M13-mp7L2 ssDNA RF DNA replication system using *E. coli* KH3 cell extract. Shown is an autoradiograph of 1% agarose electrophoretic gel on which *in vitro* replication products have been analyzed. Reactions were carried out to the indicated time intervals as described in Materials and Methods. Markers(right)for covalently closed circular DNA (RF-I) and nicked or gapped circular DNA(RF-II) are indicated.

Site-specifically modified ssDNA template for in vitro replication

The main purpose of this work is to develop an *in vitro* replication system in which M13 ssDNA bearing site-specific lesions is converted to circular duplex DNA with the ultimate goal of using such a system to identify and purify inducible factors required for translesion DNA synthesis. The strategy of the construction of εC-DNA, described previously[13], is summarized in Fig 2B. Because the 57-mer

oligonucleotide scaffold used in the construction process is complementary to the lesion-containing region of the DNA construct, any residual scaffold may serve as a primer for DNA synthesis *in vitro* such that the resulting products may complicate the analysis of the products of translesion DNA synthesis. To completely remove the scaffold we used a QIAGEN-tip 20 resin column to purify the DNA construct. In this procedure, oligonucleotides were washed out with a buffer containing 1.0 *M* NaCl at pH 7.0, and the DNA constructs(approximately 7,300 nt-long ssDNA molecules) were then eluted with a buffer containing 1.25 *M* NaCI at pH 8.5. The removal of 57-mer scaffold from DNA construct was verified by the following approach. Because the initiation of replication from M 1 3 minus strand origin requires RNA polymerase to svnthesize an RNA primer, replication of M13 ssDNA is sensitive to rifampicin, an inhibitor of RNA polymerase. In our assays, DNA synthesis from M13 reference DNA (Ml3mp7L2) as well as from QIAGEN-tip20 purified DNA constructs(εC-DNA and its control C-DNA) was undetectable if 100μg/ml rifampicin is present in the reaction,whereas only about half amount of DNA synthesis was inhibited by rifampicin when using unpurified DNA construct as template(data not shown).

Replication of εC-containing DNA with E. coli cell extracts

The replication of εC-containing DNA construct by E. coli KH2(*recA*+)and KH2R(△*recA*)cell extracts are shown in Fig 4A. For the control DNAs, approximatelv 40% of the replication products are Form I(lane 1,2, and 4). For εC-DNA, less than 5% are form I(lane 3,5), suggesting that most of replication events may be blocked due to the existence of εC lesion, but that some translesion synthesis does take place. No significant difference was observed between the replications ot εC-DNA in the two cell extracts of KH2(*recA*+)and of KH2R(△*recA*)strains, suggesting that the detected translesion synthesis did not require the RecA protein.

Because in our DNA construct the lesion is located near the terminus of replication (Fig 2), we cannot distinguish between the replication products arrested at the lesion site and those carrying only a nick due to limited DNA polymerase I, ligase, and/or gyrase activities. In both cases, DNA with a mobility of RF-II DNA will be observed in agarose electrophoretic gels. To measure translesion synthesis, we subjected the replication products to double digestion with restriction endonucleases AvaII and Ec047III. As shown in the map of M13 DNA in Fig 2, the size 0f linearized DNA by single cut is 7.26 kb, whereas the double cut will give rise to two bands with the lengths of 4.38 kb and 2.88 kb which are well-separated from each other in a 1%agarose gel. Replication block at the lesion site will produce products that are uncleavable by AvaII. The AvaII site can only be generated by DNA synthesis past the site of the lesion. Fig 4B shows an example of such an analysis of the replication products. Quantitative data averaged from two expertments are listed in Tab 1. For Control DNAs(both mp7L2 and CDNA), the fraction of about 80%of the DNA was doubly cleaved, suggesting that only a minor frac-

tion of molecules remained incompletely replicated (or degraded). In contrast, a majority of the products obtained by replicating εC-DNA was not AvaII-cleavable. Translesion synthesis was nevertheless observable to the tune of about 14% of the replicated molecules, and was found to be similar in recA and in △ *recA* cell extracts.

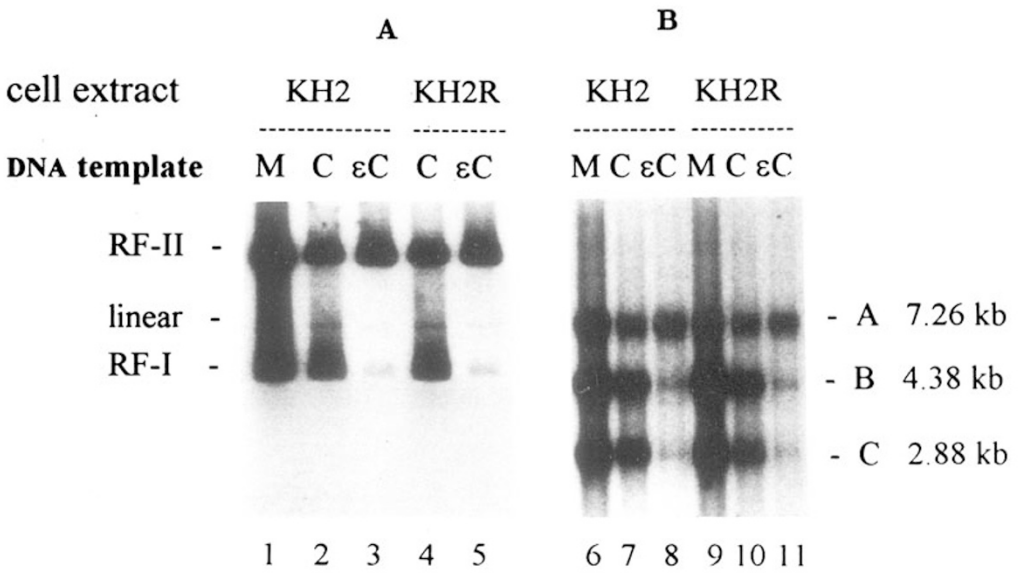

Fig 4. Effect of C lesion on the replication of M13 ssDNA in cell extracts from *E. coli* KH2 (*recA*$^+$) and KH2R (*recA*△) strains. (A) - Autoradiograph of an agarose electrophoretic gel on which replication products from different ssDNA templates have been analyzed. M, M13mp7L2; C, DNA construct containing normal cytosine in place of εC (i.e., C-DNA); εC, DNA construct containing a site-specific εC residue (εC-DNA). Procedures for the *in vitro* replication reaction (incubation for 90 min), and conditions for the gel electrophoresis are described in Materials and Methods. The positions of different forms (RF-I, RF-II) of replication products are indicated at left. (B) Restriction endonuclease analysis of the replication products. The replication products were digested by *AvaII* and *Eco* R47III (37℃ for 1 h at a 10-fold enzyme excess) and analyzed by agarose gel electrophoresis. The length of each band is extrapolated from the "1 kb marker DNA ladder" co-electrophoresed on the same gel.

Although in the first step of the construction of lesion-containing ssDNA template we examined the complete linearization of the precursor M13mp7L2 ssDNA by agarose gel, there still was apossibility that a very small fraction of uncut M13mp7L2 ssDNA molecules remained in the final DNA construct mix. If so, the efficiency of translesion synthesis detemined using the DNA construct may be overestimated. To

test this possibility, we performed another control experiment in which the replcation products were subjected to double digestion by PstI and Eco47 III. As shown in Fig 2, M13mp7L2 DNA has a unique PstI site in its polylinker region which was removed and replaced by a 17-mer sequence without PstI site in the DNA constructs. As a result, *in vitro* replication of M13mp7L2 uncut DNA is expected to produce PstI sensitive molecules; conversely, replication products of lesion-containing DNA will be resistant to PstI digestion. Gel analysis revealed that the majority of the replication products from M13mp7L2 can be cleaved into two smaller fragments (4.04 and 3.22 kb) by PstI and Eco47III. In contrast, digestion the replication products from the constructs (C-and εC-DNA) with these two enzymes gave rise to only one linear band with whole length (data not shown), suggesting that the analysis for translesion synthesis using the lesion-containing DNA construct is reliable.

Tab 1. Restriction endonuclease analysis of DNA synthesis *in vitro*

Strain for extract	DNA template[b]	DNA synthesis[b]		
		Total	Band B+C	%bypass
KH2(*recA*+)	M	528	386	73
	C	388	303	78
	εC	145	20	14
KH2R(Δ*recA*)	M	683	548	80
	C	352	288	82
	εC	118	17	14

[a]M, M13mp7L2 ssDNA; εC, ssDNA construct bearing a site-specific εC lesion; C, control ssDNA construct in which normal cytosine replaces εC.
[b] Autoradiographs in Fig 4B and those from other similar experiments were scanned with a computing densitometer, and the data on band signal density were averaged. The numbers (arbitrary density signal counts) under "Total" DNA synthesis column were derived from the sum of intensities of the three bands in each lane (i.e., A+B+C). Numbers under the "band B+C" column represent the fraction of replication products that contained the AvaII site as a result of translesion DNA synthesis, and are the sum of intensities of the 4.38 kb and 2.88 kb bands obtained by double digestion (see Fig 2A). Percent bypass is derived from the equation [B+C]/[A+B+C]. No correction factors have been applied because the lesion is located close to the terminus of DNA replication.

DISCUSSION AND PERSPECTIVE

This study was initiated to determine the effects of a single mutagenic lesion placed at a specific location of M13 ssDNA template on the DNA replication catalyzed by E. coli cell extract. Building on other available systems for in vitro DNA replication we have optimized conditions suitable for the bacterial strains and DNA substrates used in our model system. Our data indicate that the presence of a single εC lesion diminishes DNA synthesis by 80-90%. However, significant translesion synthesis (to the tune of about 10-20%) does occur, and is similar in cell extracts from *E. coli recA+* as well as △*recA* strains. These results are strikingly similar to

those observed *in vivo* after transfecting εC-DNA into competent *E. coli* cells. In the transfection experiments, survival of εC-DNA was about 11-17% of that of C-DNA in uninduced *recA+* and △*recA* cells[13]. Although we have not yet characterized the mutagenic effects of *in vitro* translesion synthesis at εC, our results so far are consistent with the notion that εC is a recA independent noninstructive lesion[10]. Although partial DNA repair can also account for the observed translesion synthesis, it is unlikely that excision repair can account for this result because the template DNA is single-stranded. However, we cannot exclude the possibility that εC was repaired to C by a *non-excisive* repair mechanism in a fraction of the molecules. If so, such a repair activity must be contained in the cell extract, and will represent a previously undescribed type of repair.

The data presented here represent our initial results using site-specifically modified DNA substrates to examine the effect of a DNA lesion on the efficiency and fidelity of replication in an *in vitro* system based on cell extracts. It is our hope that the refinement of this system will enable us to reconstitute inducible mutagenic responses *in vitro*. We are specifically interested in analyzing UVM, a recently described SOS-independent inducible response in which mutation fixation opposite εC borne on transfected M13 ssDNA is greatly enhanced by pretreatment of *E. coli* cells with DNA damaging agents. Emerging evidence indicates that the organization of the replication fork as well as the constitution of the replicative polymerase is similar in prokaryotes and eukaryotes[28, 29]. Therefore, it should be possible to extend our experimental system to model translesion DNA synthesis by the eukaryotic replication apparatus. Such a model system offers a means for identification and purification of factors required for mutagenic bypass of carcinogen-induced DNA lesions.

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