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Defective Bypass Replication of a Leading Strand Cyclobutane Thymine Dimer in Xeroderma Pigmentosum Variant Cell Extracts[1]

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ABSTRACT

Xeroderma pigmentosum variant (XP-V) is an inherited disorder resulting in hypersensitivity to the cytotoxic, mutagenic, and carcinogenic effects of UV light. There is evidence suggesting that XP-V cells carry a defect in the replication of UV-induced DNA damage, leading to mutations in genes, *e.g.*, proto-oncogenes and tumor suppressor genes, of exposed skin cells. Using an *in vitro* assay to quantitatively evaluate replication of the most prevalent UV-derived DNA lesion, the *cis,syn*-thymine dimer (T◇T), we have recently found that a T◇T located on the leading strand can be bypassed by a bona fide human replication fork but can also induce fork uncoupling with selective synthesis of the undamaged lagging strand (D. Svoboda and J-M. Vos, Proc. Natl. Acad. Sci. USA, *92:* 11975–11979, 1995). We now report the application and further refinement of this sensitive assay to the replication of a T◇T-containing template by XP-V cell-free extracts. In comparison to normal controls, a 10–26-fold deficiency in the bypass replication of T◇T was observed in XP-V cell extracts. In contrast, the disease extracts were as competent as controls for replication of the undamaged TT plasmid and for leading T◇T-induced fork uncoupling. Besides mismatch repair and nucleotide excision repair, the bypass replication defect of XP-V may represent a novel category of hereditary mutator phenotypes affecting DNA damage processing.

INTRODUCTION

XP[4] is an autosomally inherited genetic disorder that is characterized by extreme sensitivity to sunlight, as manifested by erythema, freckles, xerosis, and scaling of the skin. In addition, XP individuals exhibit the hallmark symptom of a DNA damage-processing disease, *i.e.*, "a marked predisposition to skin cancers developing after exposure to sunlight," involving a "unique conjunction of environmental, genetic, and biochemical factors in the etiology of cancer" (1). For affected XP individuals, the average age of onset of skin cancer is earlier than normal, and the probability of contracting skin cancer is ~2000 times greater than the normal risk (1). As with classical XP, XP-V patients manifest increased rates of skin cancer, and cells are hypersensitive to the cytotoxic and mutagenic effects of UV irradiation (2, 3). Nonetheless, XP-V differs strikingly from classical XP in that cells are completely normal with respect to nucleotide excision repair (2). Early investigations of XP-V, therefore, focused on the possibility of aberrant DNA replication following treatment with DNA-damaging agents (4). The XP-V defect has been postulated to be in the elongation phase of replication subsequent to UV irradiation (5) because XP-V cells are characterized by a greater than normal delay in completing replication of blocked replicons after UV irradiation (4), as well as reduced bypass replication of damaged DNA, measured at the level of individual genes (6). In addition, an increased mutation rate with unusual mutation spectrum was observed in XP-V cell lines, indicating a damage-dependent mutator phenotype (7). *In vitro* studies with SV40-based minireplicons carrying randomly distributed damage have indicated that translesion bypass of the cyclobutane pyrimidine dimers or psoralen monoadducts could be detected in normal human cell extracts (8–10). The combination of an appropriate template carrying the SV40 origin of DNA replication with nucleotides, cell extract, and a single SV40 protein, large T antigen, yields completely resolved daughter molecules derived from a bidirectional, semiconservative replication mechanism (11–13). We and others have used a DNA template containing a single, defined T◇T to make a detailed quantitative and mechanistic evaluation of the replication of this predominant UV-damaged DNA lesion by a bona fide replication fork generated in extracts from normal human cells (14–16). This system specifically defines the effect of a strand-specific, uniquely located UV lesion on the completion of replication, at the level of nascent strand elongation (17). We now report the application and further improvement of this sensitive assay to compare *in vitro* bypass replication of a single T◇T by normal and XP-V cell-free extracts.

MATERIALS AND METHODS

Cell Extract and Replication Bypass Assays. HeLa, VA13, and GM02449B cells were obtained from the American Type Culture Collection (Camden, NJ). HSC93 cells were obtained from M. Buchwald (Hospital for Sick Children, Toronto, Ontario, Canada). CTAG and SV80 cells were obtained from W. Kaufmann and M. Cordeiro-Stone (Department of Pathology, University of North Carolina at Chapel Hill; Ref. 18). Cells were grown in SMEM (suspended cells; Life Technologies, Inc.) or DMEM (attached cells; Life Technologies, Inc.), containing 5% (HeLa) or 10% (VA13, GM02449B, CTAG, and SV80) fetal bovine serum (HyClone). Preparation of the template DNA, cell-free extracts, and incubation conditions were as described (14, 17). Replication activities on undamaged TT plasmid of extracts from the various cell lines were similar, within a factor of 2 (data not shown). Conditions for agarose gel electrophoresis and band quantitation for the calculation of the efficiencies of bypass replication and fork uncoupling have been described elsewhere (14, 17). Briefly, 25-ng samples of template were incubated in cell extracts at 37°C for the appropriate times, and replication products were analyzed by incubation with T4 UV endo and electrophoresis on a native agarose gel. The amount (in PhosphorImager units) of form I DNA, templated by the T◇T-containing template strand (*i.e.*, T4 UV endo-sensitive, evaluated by measurement of the disappearance of form I due to its conversion to form II) at each time point was calculated from the intensities of bands on the gel.

Mismatch-containing T◇T Template and Assay. Where indicated, undamaged and T◇T-containing template carried a single mismatch at the unique *Mfe*I site. The pKSoriD-*Mfe*I (T◇T-containing plasmid and TT-containing control) were prepared by annealing the original undamaged or T◇T-containing 20-nucleotide oligomer (17) to a circular, single-stranded DNA derived from pKSoriD(−) (14) by a single nucleotide change at the position 1293 (T to C), creating a unique *Mfe*I site opposite the TT oligonucleotide sequence. This resulted in a synthetic, double-stranded DNA molecule with a unique *Mfe*I site in the undamaged strand opposite the T◇T (or TT in the control template; Fig. 2*a*). The single mismatched bp leaves the T◇T (or TT) strand without the *Mfe*I site.

In addition, the templates were treated *in vitro* with dam methylase, yielding fully methylated GATC sites. After replication in human cell extract, the daughter molecules synthesized from the undamaged strand were sensitive to *Mfe*I digestion, whereas daughter molecules from the T◇T-containing strand

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[4] The abbreviations used are: XP, xeroderma pigmentosum; XP-V, XP variant; endo, endonuclease.

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(or TT-containing control) were refractory to *Mfe*I digestion. Background label-containing form I DNA generated from nonspecific incorporation of short patches of nucleotides (without T antigen) was removed by digestion of all samples, with the methylation-specific *Dpn*I endo yielding small fragments. Second-round daughter molecules were digested with *Mbo*I, which only digests DNA that is unmethylated on both strands. Treatment with *Mfe*I linearized daughter form I molecules, synthesized from the undamaged strand, leaving daughter molecules synthesized from the T◇T-containing strand as the sole, label-containing form I molecules. Replication of the T◇T-containing template strand was revealed by the presence of a form I gel band following the *Mfe*I/*Dpn*I/*Mbo*I restriction enzyme digestions. Complete digestion with T4 UV endo confirmed that the only labeled form I DNA remaining was the replicated T◇T-containing template. The level of T◇T-replication was then expressed as the ratio of the intensity of the T4 UV endo-sensitive band to the total form I DNA synthesized.

RESULTS

Time Course of the Synthesis of T◇T-containing DNA. The result of replication of the SV40-based double-stranded DNA pKSoriD(−) template, carrying a single T◇T incubated in cell extracts from normal (VA-13) or XP-V (CTAG) cells, was analyzed by agarose gel electrophoresis after digestion with T4 UV endo, which specifically nicks DNA at cyclobutane pyrimidine dimers (Fig. 1). Fig. 1*a* illustrates the reduced replication level, *i.e.*, form I synthesis, and relative bypass level, *i.e.*, fraction of T◇T-containing form I, observed with T◇T-modified template DNA incubated in normal and XP-V extracts. The fraction of form I replication product nicked by T4 UV endo was reduced in the XP-V extract compared to normal. In contrast, the amount of fully replicated molecules, *i.e.*, form I synthesis, from undamaged TT plasmid was similar in the XP-V and normal extracts. As shown below, these observations indicated that bypass replication of the T◇T lesion was selectively impaired in XP-V extracts. In addition, the amount of form I synthesis from damaged T◇T plasmid was only slightly lower in XP-V extract than in the normal ones. This indicated that selective synthesis of form I from the undamaged strand by fork uncoupling was preserved in the disease extracts. Quantitation of the amount of fully replicated T◇T-containing daughter molecules relative to undamaged template over the incubation times in these extracts confirmed the reduced bypass synthesis of T◇T in XP-V (Fig. 1*b*). The average bypass replication efficiency over time was 16 ± 4% for normal but only 2.1 ± 1% for XP-V cell extracts, resulting in a 7.7-fold reduction in bypass efficiency in the disease cell extracts.

T◇T Replication with Normal and XP-V Cell Extracts: Selective Digestion of Undamaged Daughter Molecules at Latent *Mfe*I Site. As outlined above and in Fig. 2*a*, a template carrying a one base mismatch at a unique *Mfe*I restriction site allows selective linearization of the replication products made from the undamaged strand, while leaving intact the replication products made from the damaged strand. Hence, this strand-specific *Mfe*I assay can distinguish bypass replication and fork uncoupling. As shown in Fig. 2*b*, incorporation of a latent restriction site (*Mfe*I) in the undamaged strand of the template, together with a template with both strands methylated at GATC sites (dam methylase), resulted in the direct observation of T◇T-containing form I product DNA. Although a substantial fraction of form I made in normal extracts was resistant to *Mfe*I cleavage but completely sensitive to cleavage by the T◇T-specific UV endo, there was no visually detectable *Mfe*I-resistant form I replication product made in the two different XP-V extracts. In contrast to the normal cell extracts, nearly 100% of the fully replicated T◇T-containing plasmid made in the disease cell extracts resulted from the selective replication of the undamaged strand, *i.e.*, fork uncoupling. Quantitation of the gel images resulting from various normal and XP-V cell extracts gave the efficiency of bypass replication on T◇T-containing template relative to undamaged template (Fig. 2*c*). Comparison of normal cell extracts from several sources with XP-V extracts from both transformed fibroblast and lymphoblastoid cell lines derived from different patients yielded a 10–26-fold deficiency in the completion of form I DNA synthesis templated by the T◇T-containing strand. In contrast, both the efficiencies of replication on undamaged TT plasmid and of

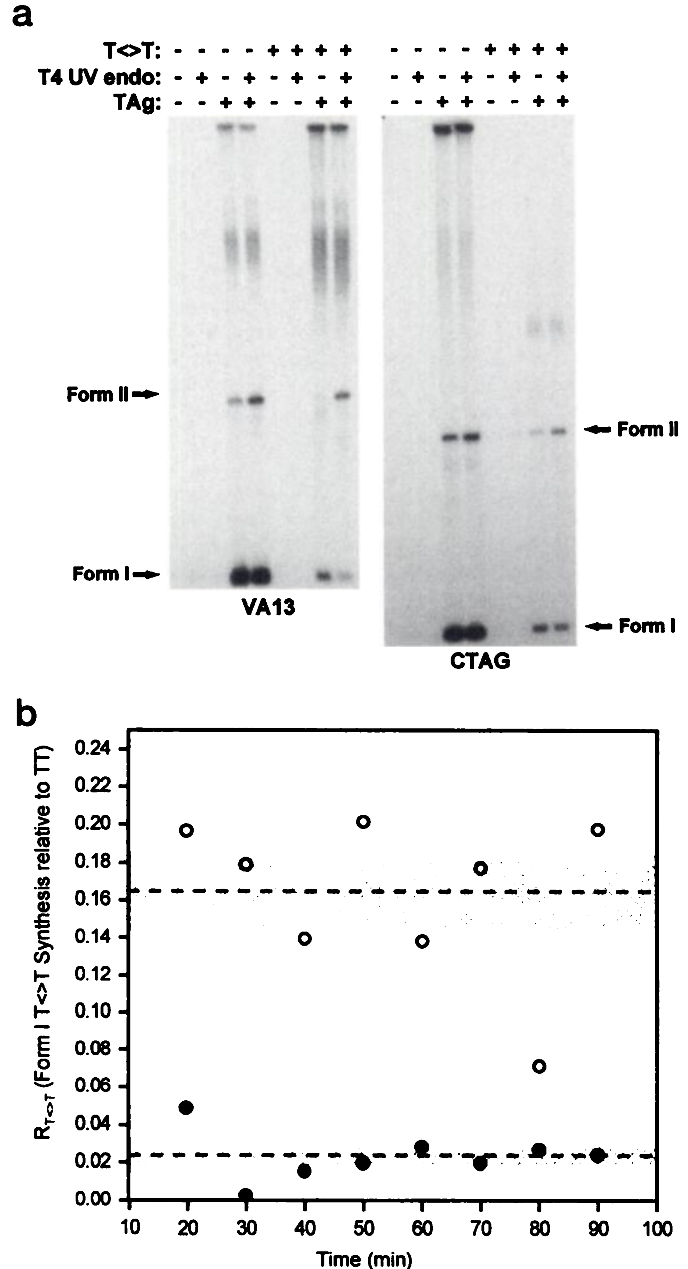

Fig. 1. Defective bypass replication of a UV-induced cyclobutane thymine dimer in XP-V cell-free extracts. *a*, agarose gel analysis of replication of undamaged and T◇T-containing templates *in vitro* with normal (VA13) and XP-V (CTAG) fibroblast cell extracts. Semisynthetic plasmid DNA molecules containing a single T◇T dimer were incubated with VA13 or CTAG cell-free extracts for 60 min, and labeled replication products were treated with the T4 UV endo, followed by agarose gel electrophoresis. Labeled form I DNA containing T◇T as nicked by the T4 UV endo and migrated as form II. *b*, comparison of bypass synthesis using normal VA13 fibroblast (○) and XP-V CTAG fibroblast (●) as a function of time. The level of replicated T◇T template is expressed as the ratio ($R_{T◇T}$) of the amount of T◇T-specific replication (form $I_{T4\text{-sensitive}}$) to the amount of replication with the control (TT) template ($R_{T◇T}$ = form $I_{T4\text{-sensitive}}$/form I_{TT}). Comparison of this ratio between the two cell extracts gives a measure of the extract's relative competence in T◇T bypass replication (17). - - - - -, means; ▨, SD.

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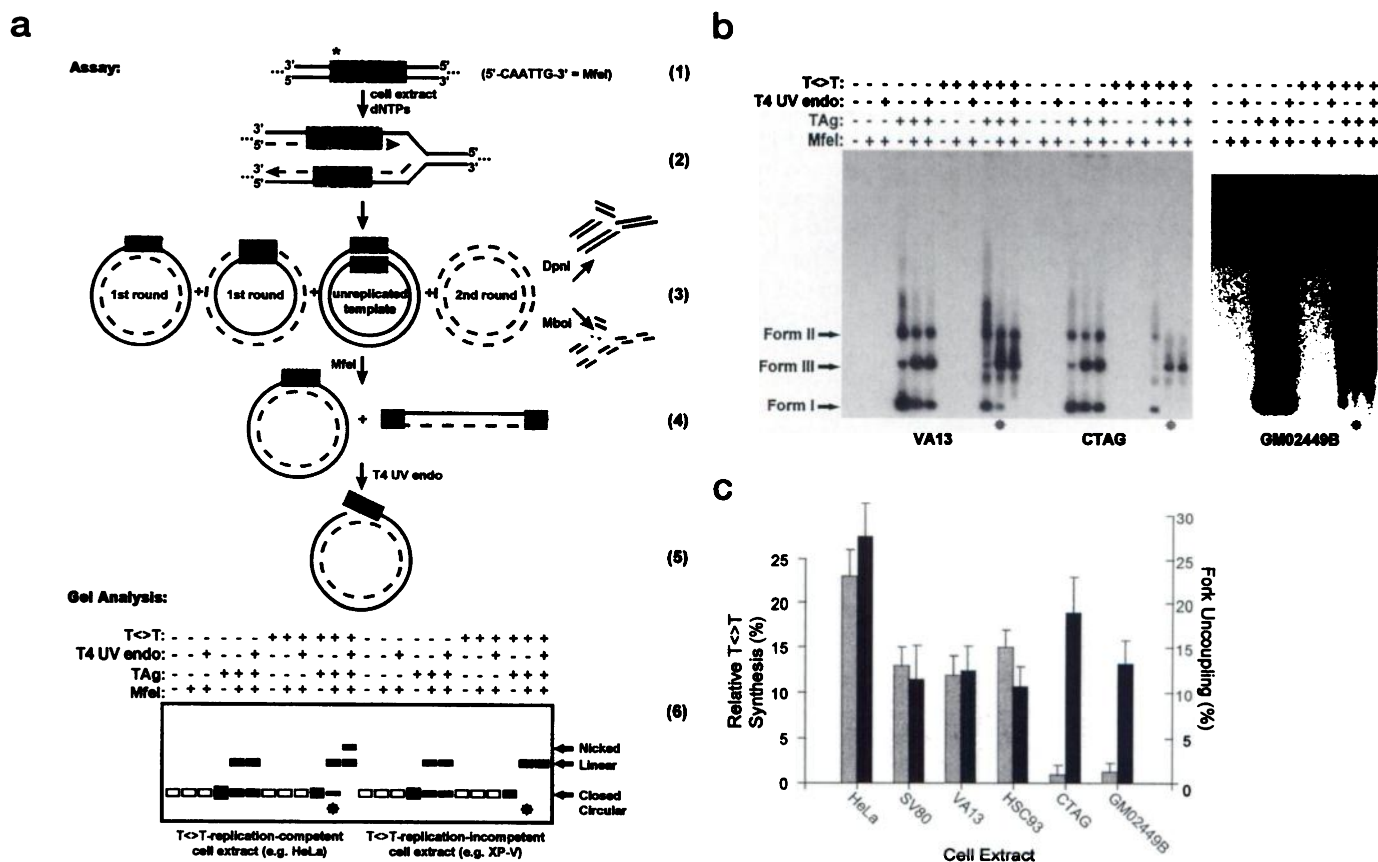

Fig. 2. Defective T◇T bypass replication but proficient fork uncoupling in cell-free extracts from different XP-V patients. *a*, schematic strategy of the fully methylated strand-specific latent *Mfe*I template assay to analyze T◇T bypass replication and fork uncoupling. The cartoon diagram of an idealized autoradiograph shows the expected pattern of bands resulting from replication of the T◇T-containing template strand. After *MfeI/DpnI/Mbo*I restriction enzyme digestions and T4 UV endo treatment, the residual form I DNA is derived from replication of the T◇T-containing strand (*) for both normal and T◇T replication-deficient cell extracts. *b*, comparison of T◇T bypass replication using the strand-specific *Mfe*I mismatch template between normal and XP-V cell-free extracts. Autoradiograph of 60-min replication incubations illustrating the defective production of T◇T-containing form I molecules in extracts prepared from two different XP-V patients and cell types. The VA13 control (also SV80 and HSC93; gels not shown) results in efficient replication of T◇T (*), whereas transformed fibroblast XP-V (CTAG), and lymphoblastoid XP-V (GM02449B) yield an extremely weak T◇T replication signal (*). *c*, histogram of the quantitation of the levels of T◇T bypass replication among the different normal and XP-V cell extracts tested. The bypass replication data are represented as the ratio of T◇T-containing form I DNA from the T◇T template to total form I DNA from the control template (*left axis*), whereas the fork uncoupling data are shown as the ratio of T◇T-free form I DNA from the T◇T template to total form I DNA from the control template (*right axis*). *Columns*, means from two to five experiments; *bars*, SD.

fork uncoupling on damaged T◇T plasmid occurred to a similar extent in the XP-V extracts as observed in normal extracts.

DISCUSSION

Previously, we developed a method that, for the first time, allowed direct, strand-specific quantitation of the rate of replication of a T◇T-containing template *in vitro* with a bona fide human replication fork (14). This assay, while indicating a significant deficiency in the replication of T◇T when applied to XP-V cell extract, is complicated by the presence of fully replicated molecules derived from "uncoupled" synthesis, along with product from the damaged strand. Therefore, calculation of the relative levels of T◇T replication requires time course experiments, followed by data analysis involving subtractions of large background values, *i.e.*, spontaneously nicked form II and nonspecific nicking by the UV endo (14). Hence, visual inspection of an autoradiogram, such as that in Fig. 1*a*, is not effective at rapidly and accurately detecting a bypass deficiency in the XP-V extracts, a prerequisite for the fractionation and purification of an XP-V complementing activity from normal human cell extract. We, therefore, designed a new template with improved signal:noise ratio to obtain a clearer visual indication of the level of T◇T replication by autoradiography of agarose gels using a single time point (Fig. 2, *a* and *b*).

Because replication efficiency on undamaged TT plasmid is similar in XP-V and normal extracts, we conclude that the disease cells carry a specific defect in T◇T bypass replication, providing a direct demonstration of the hypothesis proposed more than 20 years ago (4). The data from the improved strand-specific *Mfe*I enzymatic assay for the analysis of replication products confirm the relative rate of leading strand T◇T replication (*i.e.*, ~20%) that was previously reported in normal human cell extracts (14). The extreme reduction in bypass replication of a T◇T selectively located in the leading strand template (Figs. 1 and 2), together with the partial reduction of psoralen bypass observed in an housekeeping gene of XP-V cells (6), could suggest that the XP-V defect is specific to the leading strand. In support, a recent report using a similar SV40-based *in vitro* replication system combined with analysis by two-dimensional gel electrophoresis of replication intermediates documented a severe impairment of leading dimer bypass in cell extracts from XP-V fibroblasts (16). However, preliminary results with a lagging strand T◇T template *in vitro* resulted in a replication defect similar to the leading strand (17).

Synthesis of the undamaged strand due to fork uncoupling occurs to a similar extent in normal and XP-V extracts (Fig. 2*c*), at values similar to those in our previous report using normal human cell extracts (*i.e.*,~20%; Ref. 14). Given the profoundly reduced rate of T◇T replication in XP-V and the ability of XP-V cell extracts to complete synthesis of the undamaged strand through fork uncoupling,

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long-lived single stranded regions opposite T◇T can be predicted to occur in UV-exposed XP-V cells *in vivo*. The size of these gaps *in vitro* remains to be established, although one would hypothesize that they may be at least of the size of an Okazaki fragment; ~300 nt for the SV40 *in vitro* system (11–13). In the SV40-based system, the concerted action of at least 22 polypeptides is required to accomplish chromosomal DNA replication, followed by decatenation and supercoiling of the daughter molecules (11–13). Several of the known replication factors may be rationalized as candidates for XP-V-correcting factor: (*a*) DNA polymerase δ catalyzes elongation of the primed template and, therefore, interacts directly with T◇T during nucleotide insertion; (*b*) proliferating cell nuclear antigen acts as a molecular clamp, reducing the rate that polymerase δ dissociates from the primer end, increasing its processivity; and (*c*) RF-C facilitates the loading of polymerase delta onto primer ends. These three candidates possess activities that influence the resultant rate of nucleotide insertion at the level of catalysis, polymerase dissociation, or polymerase binding. Another attractive possibility is the potential presence of an accessory factor that facilitates translesion synthesis analogous to the bacterial umuCD (19, 20) and yeast REV systems (21). Isolation of the XP-V-complementing activity should help to reveal the biochemical mechanism of replication of UV-damaged DNA templates and the mechanism of mutation induction by T◇T in human cells.

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