Bypass of a Site-Specific Cis-Syn Thymine Dimer in an SV40 Vector during in Vitro Replication by HeLa and XPV Cell-Free Extracts†

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ABSTRACT: The key step in skin cancer induction by UV light is thought to be the mutagenic DNA synthesis past a DNA photoproduct in a proto-oncogene or tumor suppressor gene. To investigate this critical step, we have constructed an SV40 vector containing a cis-syn thymine dimer, the major DNA photoproduct induced by UVB light, within an *Ase*I site at a location that would initially be replicated by leading strand synthesis. When the dimer-containing SV40 vector was incubated with cell-free HeLa extracts in the presence of TAg, and then digested with *Ase*I, a 2325 bp fragment corresponding to inhibition of cleavage at the dimer site was observed, suggesting that the dimer had terminated synthesis and/or had been bypassed. When the reaction was limited to one round of replication and the products of restriction enzyme digestion were examined by denaturing gel electrophoresis, bands corresponding to both termination and bypass were observed in roughly a one-to-one ratio. Whereas increasing the dNTP concentration from 10 μM to 1 mM increased the ratio of bypass to termination from 0.6 to 2.6, it had no effect on the site of termination, which occurred exclusively one nucleotide before the dimer. Experiments in which dGTP was held constant at 25 μM and various combinations of the remaining nucleotides were raised from 25 μM to 1 mM showed substantial increases in the bypass-to-termination ratio, with the greatest effect seen for raising all three nucleotides to 1 mM. Replication by primary fibroblast XPV extracts was also investigated and found to be greatly stimulated by rhRPA, whereas the stimulatory effect for HeLa cell extracts was variable. In the presence of rhRPA, the XPV extracts were also found to bypass the cis-syn dimer, which contrasts with a recent report that could not detect dimer bypass in SV40 transformed XPV extracts in the absence of added replication factors [Cordeiro-Stone, M., et al. (1997) *J. Biol. Chem. 272*, 13945−13954].

The key step in skin cancer induction by UV light is thought to be the mutagenic synthesis past a DNA photoproduct in a proto-oncogene or tumor suppressor gene (*1*, *2*, *3*). Despite much research in this area, little is known about the precise structure−activity relationships in mutagenesis by DNA photoproducts in human cells and the mechanism by which mutagenic synthesis past a photoproduct takes place. Simian virus 40 (SV40) and shuttle vectors containing the SV40 origin have been useful model systems for the study of the processing of DNA damage during mammalian DNA replication (*4*). A number of studies have been carried out with randomly irradiated plasmid DNA containing the SV40 replication origin, which were replicated by cytoplasmic extracts from HeLa cells in the presence of SV40 large T antigen (TAg).[1] In analogy with similar studies in prokaryotes, SV40 DNA replication was inhibited in a dose-dependent manner, and plasmids that underwent complete DNA replication were shown to have an increased number of mutations by *E. coli* transfection assays (*5*, *6*). Recently, the use of site-specifically damaged SV40 origin-containing plasmids has enormously increased our understanding in quantitative terms of the consequences of such damage to the progression of the DNA replication fork. Using SV40 vectors containing a site-specific cis-syn thymine dimer in either strand, Svoboda and Vos found that both leading and lagging strand synthesis were inhibited to a comparable extent, and that some of the synthesis became uncoupled (*7*). In another study using site-specific photoproducts, Carty et al. concluded that the (6-4) photoproduct was more inhibitory than the cis-syn dimer on lagging strand synthesis, and that both photoproducts were weakly mutagenic (*8*). In the most recent study, Cordeiro-Stone and co-workers found that leading strand synthesis in cytoplasmic extracts of SV40 immortalized Xeroderma pigmentosum variant (XPV) fi-

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[1] Abbreviations: (6-4) photoproduct, (6-4) pyrimidine−pyrimidone photoproduct; *dam*, *E. coli* DNA-adenine-methylase; di, *dam* methylated cis-syn cyclobutane thymine dimer-containing pSVK3; di$^-$, unmethylated cis-syn cyclobutane thymine dimer-containing pSVK3; DTT, dithiothreitol; dNMP, deoxynucleotide monophosphate; dNTP, deoxynucleotide triphosphate; nt, nucleotide; PCNA, proliferating cell nuclear antigen; RF, replicative form; RF-I, supercoiled form; RF-II, nicked or open circular form; RF-IV, relaxed closed circular form; rhRPA, recombinant human single-stranded DNA binding protein; RSP, enzymatic digestion with *Rsa*I, *Sty*I, and *Pvu*II; RPSA, enzymatic digestion with *Rsa*I, *Sty*I, *Pvu*II, and *Ase*I; SV40, simian virus 40; TAg, SV40 large T antigen; wt, *dam* methylated wild-type pSVK3; XPV, Xeroderma pigmentosum variant.

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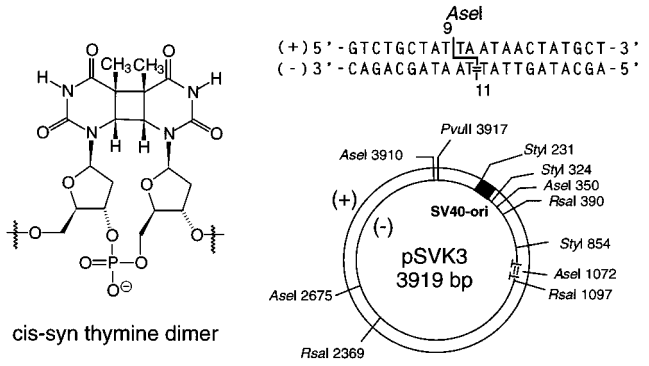

FIGURE 1: The structure of the cis-syn thymine dimer and the oligonucleotide and vector into which it was incorporated for in vitro replication studies. The site of the dimer in the 22-mer primer and pSVK3 is indicated by T=T. Also shown is the 22-mer complementary to the primer that was used in characterizing the site and integrity of the dimer. Sites of the restriction enzymes used in the RSP and RSPA digestion assays are also indicated.

broblasts was severely impaired by a site-specific cis-syn dimer (*9*). At the present time, the precise molecular defect in XPV is unknown, but it appears to be in the pathway associated with post replication repair (*10*, *11*).

In a previous in vitro study with site-specific photoproduct-containing templates, we have shown that DNA polymerase δ (polδ) from calf thymus cannot synthesize past a cis-syn or a trans-syn thymine dimer, unless PCNA is also present (*12*). A similar result was also recently reported for synthesis opposite an abasic site analogue (*13*). The termination site for polδ at the cis-syn dimer in the absence of PCNA was one nucleotide before the dimer at all dNTP concentrations. In the presence of PCNA, the major termination site was one nucleotide before the 3′-T of the dimer at 1 μM dNTPs and opposite the 5′-T of the dimer at 100 μM dNTP concentrations. Whereas little synthesis past the photoproduct was observed at low dNTP concentrations in the presence of PCNA, raising the dNTP concentration to 100 μM led to significant synthesis past the cis-syn dimer. We now report the effect of dNTP concentration on the bypass of a site-specific cis-syn thymine dimer in an SV40 vector by HeLa cell extracts during leading strand synthesis. Whereas increasing the dNTP concentration was found to increase the amount of bypass of the dimer in the SV40 vector, it does not appear to affect the site of termination as it did with calf thymus polδ in vitro. In contrast to a recent study conducted with SV40 transformed XPV cell lines in the absence of added replication factors (*9*), we find that extracts from a primary fibroblast XPV cell line can bypass a cis-syn thymine dimer in the presence of recombinant human RPA.

MATERIALS AND METHODS

Materials. The cis-syn thymine dimer-containing 22 nt oligonucleotide (Figure 1) was synthesized by the building-block method (*14*). T4 *denV* endonuclease V was obtained from R. S. Lloyd (UTMB, Galvaston, TX), and *Escherichia coli* photolyase was obtained from A. Sancar (UNC, Chapel Hill, NC). The SV40 origin-containing vector pSVK3 was purchased from Pharmacia (Piscataway, NJ). HeLa cells were purchased from Cellex Biosciences, Inc. (Minneapolis, MN), and primary untransformed Xeroderma pigmentosum variant (XPV) fibroblast cells, GMO2359 were obtained from Coriell Cell Repositories, (Camden, NJ). Fetal calf serum was purchased from Intergen (Purchase, NY), and tissue culture flasks were supplied by Becton, Dickenson (Franklin Lakes, NY). Human recombinant single-stranded DNA binding protein (rhRPA) was purified according to Hendricksen et al. (*15*) from an expression vector provided by M. Wold (University of Iowa, Iowa City, IA). SV40 large T antigen (TAg) was purchased from Molecular Biology Resources (Milwaukee, WI), DNA adenine methylase (*dam*) from NEB (Beverly, MA), and Sequenase 2.0 from USB (Cleveland, OH). Restriction enzymes were from NEB, and other enzymes from Boehringer Mannheim.

Enzymatic Analysis of the Dimer-Containing 22-Mer. The dimer-containing 22-mer or its complementary strand (72 pmol) were 5′-end-labeled and annealed to 144 pmol of unlabeled complementary 22-mer in 12 μL of TE buffer. One-half of the annealed DNA was diluted into 14.5 μL of 100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM DTT, 1 mM EDTA, 0.1 mg/mL BSA, 4% glycerol, and 4 pmol of *E. coli* photolyase. The solution was irradiated with 366 nm light for 30 min on ice, ethanol precipitated, and redissolved in 6 μL of TE buffer. One μL aliquots of either photolyase treated or untreated solutions containing 6 pmol of labeled and 12 pmol of unlabeled 22-mer were then treated with piperidine, T4 *denV* endonuclease V with or without subsequent piperidine treatment, or with *Ase*I. Piperidine treatment was carried out in 100 μL of freshly diluted 1 M piperidine for 30 min at 100 °C, followed by evaporation of the solution under vacuum. T4 endonuclease treatment was carried out with 32 ng of T4 *denV* endonuclease V in 10 μL of 32 mM K/HPO_4, pH 8.3, 100 mM NaCl, 10 mM EDTA, and 100 μg/mL BSA. Digestion with *Ase*I was carried out in 10 μL of 100 mM NaCl, 50 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, and 2 units of *Ase*I for 2 h at 37 °C.

Construction of an SV40-Vector Containing a Site-Specific Cis-Syn Thymine Dimer. The dimer-containing 22-mer (2220 pmol) was phosphorylated with T4 polynucleotide kinase and annealed to 222 pmol of single-strand pSVK3 DNA. The single-stranded form of pSVK3 was obtained by superinfection of pSVK3 infected *E. coli* with the helper phage R408 (*16*). Phage particles were purified by banding through a CsCl gradient, and single-strand pSVK3 DNA isolated by proteinase K digestion, phenol extraction, and ethanol precipitation as described (*17*). The primed pSVK3 DNA was extended with 150 pmol of Sequenase Version 2.0 and ligated with 16 000 units of T4 DNA ligase in a total reaction of 1 mL containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, 5 mM spermidine hydrochloride, 200 μM of each dNTP for 1 h at 37 °C. Fully ligated lesion-containing pSVK3 molecules were purified from unligated and unreplicated molecules by a CsCl ethidium bromide gradient (*17*). A portion of this covalently closed plasmid preparation was fully methylated with *E. coli* DNA-adenine-methylase (*dam*) according to the manufacturer's instructions. Wild-type pSVK3 was purified using a Qiagen Kit (Qiagen Inc., Valencia, CA), followed by a CsCl-ethidium bromide gradient as performed for the dimer-containing pSVK3.

In Vitro Replication Assays. The SV40 replication assays were carried out according to a literature procedure (*18*), as was the preparation of the cell extracts (*19*). Cytoplasmic XPV extracts (0.5 mL) were obtained from 1×10^8

exponentially growing XPV cells grown in 15% fetal calf serum in 80 75 cm^2 or 50 175 cm^2 tissue culture flasks. Unless otherwise indicated, SV40 replication reactions (25 μL) were carried out in 30 mM Hepes-NaOH, pH 7.8, 7 mM $MgCl_2$, 40 mM creatine phosphate, 0.1 mg/mL creatine kinase, 0.1 mM DTT, 4 μM ATP, 50 μM each of CTP, GTP, and UTP, 100 μM each of dCTP, dGTP, and dTTP, 25 μM of [α-^{32}P]dATP (a total of 2−4 μCi of label per assay), 50 ng (0.02 pmol) of unmodified or dimer-containing pSVK3, 1 μg of TAg, and cytoplasmic cell extract (100−120 μg of protein). The extract was spun for 5 min at 4 °C in a microcentrifuge before addition to the replication assay (M. Wold, personal communication). The reaction mix was incubated at 37 °C for 30 min or for the times indicated in the Figure legends. The reaction mixtures were then treated with proteinase K, phenol extracted, and ethanol precipitated as described (*19*). Restriction digestion was carried out in buffers recommended by the manufacturers, and analyzed by 1% agarose gel electrophoresis or denaturing 8% polyacrylamide-7 M urea gel electrophoresis. Products were quantified by either laser densitometry of an autoradiogram, or by direct phosphoImager analysis. Replication efficiencies of cell extracts were determined by quantifying the number of acid precipitatable counts according to a standard procedure (see for example, ref *20*).

RESULTS

Design of the Dimer-Containing SV40 Vector. To compare the results of our previous in vitro study with calf thymus polδ and PCNA with that of a human replication system, we needed to incorporate the dimer into a site that would be initially encountered by the leading strand replication apparatus. The commercially available pSVK3 vector has SV40, ColE1, and f1 origins allowing preparation of the (+) strand and introduction of the dimer into the (−) strand of the duplex form by dimer-containing primer extension followed by ligation (Figure 1). Thus, encounter of a dimer by the leading strand replication apparatus could be accomplished by introduction of the dimer into the (−) strand within a kilobase to the 3′-side of the SV40 origin. We decided to incorporate the dimer into the (−) strand of the *Ase*I site at 1072 nt, to ensure that the replication fork would be proceeding processively by the time it reached the dimer. This particular restriction site was also chosen because, regardless of the ability of the enzyme to cleave the phosphodiester linkages between the two T's of this site, the DNA should be completely refractory to double-strand cleavage because of the cyclobutane dimer linkage (Figure 1). To determine whether the dimer would also inhibit cleavage by *Ase*I on the undamaged complementary strand to the dimer, a 22-mer duplex corresponding to the DNA sequence at the *Ase*I 1072 site was prepared. The site and integrity of the cis-syn thymine dimer was established by the use of two cis-syn dimer-specific repair enzymes and alkalai treatment (Figure 2). Treatment of the 22-mers with hot piperidine did not lead to any specific cleavage under conditions known to cleave DNA at (6-4) products but not at cis-syn dimers (lane 2) (*21*). Treatment with the cis-syn dimer-specific T4 *denV* endonuclease V initially led to bands migrating between 10 and 12 nt which correspond to enzyme-catalyzed β-elimination products (Figure 2a, lane 3 right) (*22*, *23*). These products were then converted by hot piperidine to a faster moving band (lane 4) corresponding to the δ-elimination product (*22*, *23*), d(pAGCATAGTTAp), which migrates more like a 9-mer in the standard lane because of the presence of a 3′-phosphate (*24*, *25*). When

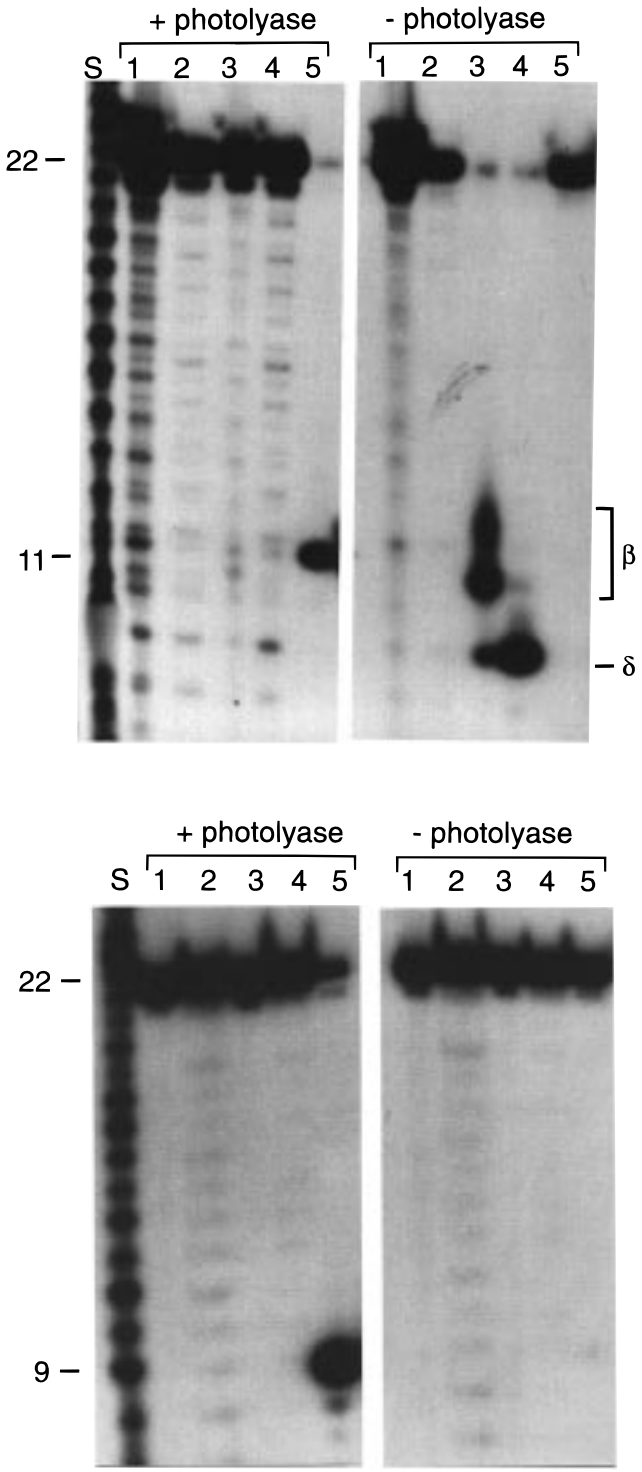

FIGURE 2: Characterization of the cis-syn thymine dimer containing 22-mer duplex. Denaturing polyacrylamide gel electrophoresis of the products of chemical and enzymatic treatment of 5′-^{32}P-end-labeled (a) dimer-containing strand, and (b) complementary strand before and after treatment with photolyase and light. Lane s, size marker; 1, untreated; 2, hot piperidine; 3, T4 *denV* endonuclease V; 4, T4 endonuclease followed by hot piperidine; 5, *Ase*I. The bands labeled β correspond to products of the β-elimination step catalyzed by the endonuclease, whereas the band labeled δ corresponds to the product of the δ-elimination step catalyzed by piperidine (see text).

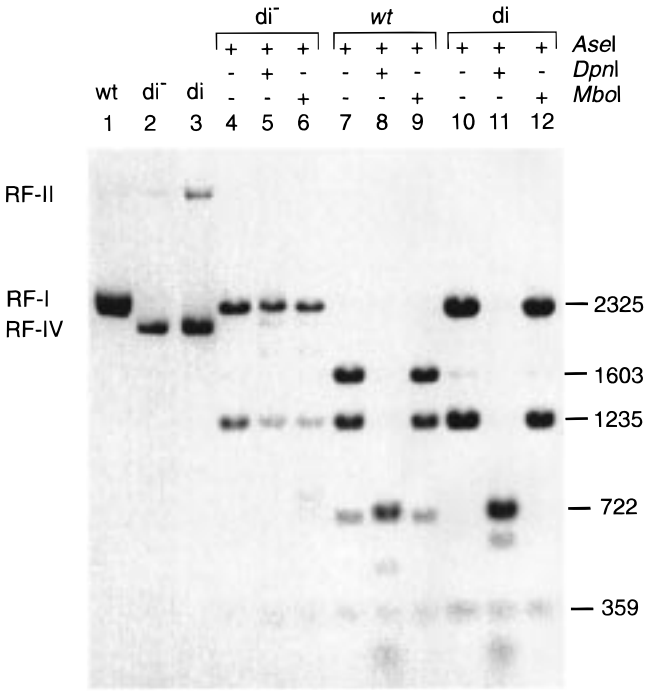

FIGURE 3: Characterization of the wild-type and dimer-containing pSVK3. Ethidium bromide-stained agarose gel (0.8%) electrophoretic analysis of *dam* methylated wild-type (wt) and methylated (di) or unmethylated (di⁻) dimer-containing pSVK3 before and after restriction digestion with different combinations of *Ase*I, *Dpn*I, and *Mbo*I. *Mbo*I can only cleave unmethylated GATC sites, whereas *Dpn*I can only cleave *dam* methylated GATC sites. What appears to be an uncleaved 722 bp fragment in lanes 8 and 11 is actually the result of *Dpn*I and *Ase*I cleavage which leads to 736 bp (3174−3910) and 735 bp (1434−2169) fragments which comigrate with the 722 bp fragment generated by *Ase*I digestion alone. The positions of supercoiled (RF-I) nicked or open circular (RF-II) and relaxed closed circular (RF-IV) forms are indicated on the left.

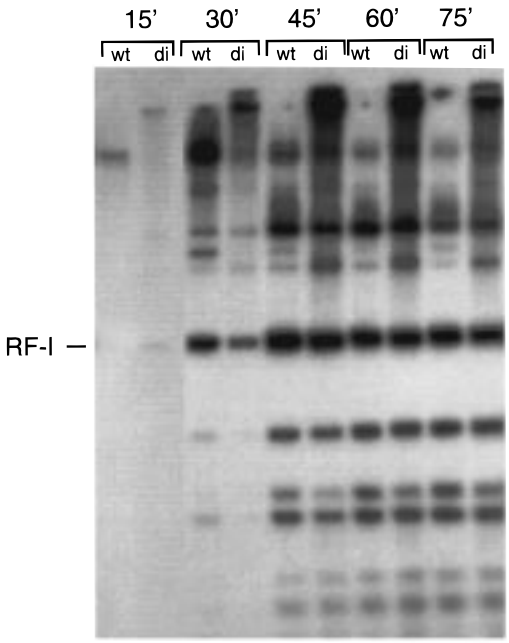

FIGURE 4: Time course of single and multiple rounds of replication of pSVK3 vectors. Autoradiogram of a 1% agarose electrophoresis gel of *dam* methylated wild-type (wt) and dimer-containing (di) pSVK3 that were replicated for the indicated times in minutes in the presence of [α-^{32}P]dATP, and then treated *Mbo*I. Products of a single round of replication are hemimethylated and appear as RF-I molecules (supercoiled form) and slower moving concatemers, whereas products of multiple rounds of replication that do not contain an original parental strand are unmethylated and thus degraded by *Mbo*I to shorter fragments.

the duplex was incubated with *Ase*I no cleavage was observed when either strand was end-labeled, but both strands could be cleaved following treatment with the cis-syn dimer-specific *E. coli* photolyase and 366 nm light. The ability of the dimer to inhibit single- and double-strand cleavage by *Ase*I could then be used to assay for repair of the dimer and to distinguish between replication products of the dimer- and nondimer-containing strands.

Construction and Characterization of the Dimer-Containing SV40 Vector. The dimer-containing plasmid was constructed by standard oligonucleotide-directed mutagenesis techniques which have been successfully used to incorporate DNA damage into replicative form bacteriophages (*26*−*28*). The presence of the dimer at the *Ase*I 1072 site could be confirmed by incubation with *Ase*I, followed by agarose gel electrophoresis. Inhibition of *Ase*I cleavage at this site results in a 2325 bp fragment, and the loss of the 1603 and 722 bp fragments (Figure 3).

Time Course of Replication. To establish the time frame in which one round of DNA replication occurs, use was made of the fact that *Mbo*I cannot cleave fully or hemimethylated GATC sites. Because human cell-free extracts have not been found to methylate DNA during replication (*29*), the replication products generated in multiple rounds are *Mbo*I sensitive. Thus, the only radiolabeled products that are *Mbo*I resistant are those that result from the first round of replication and those products of subsequent rounds of replication which incorporate one of the parental strands. Whereas the wild-type vector is fully methylated by the *E. coli* strain used, the dimer-containing vector produced by primer extension and ligation had to be enzymatically methylated with *dam* methylase. Both vectors were characterized by their reaction with the methylation-dependent restriction enzymes *Mbo*I and *Dpn*I which cleaves only at a methylated site (Figure 3). The methylated vectors were then replicated by the cell-free extracts in the presence of [a-^{32}P]dATP and TAg, and aliquots were removed at various times and digested with *Mbo*I to detect unmethylated replication products (Figure 4). Significant amounts of replication products in the form of RF-I and slower moving concatamers were only observed after 30 min of incubation. At this time point, only about 10% of the replication products of both the undamaged and dimer-containing vectors were *Mbo*I resistant.

Agarose Gel Assays of the Replication Reaction. Because the in vitro replication assays were carried out with unlabeled substrate in the presence of [α-^{32}P]dATP, only one strand of the replication products are labeled after one round of replication, whereas both strands of the products are labeled after multiple rounds of replication. The formation of hemilabeled products during the first round of replication and the ability of the cis-syn dimer to inhibit double-strand cleavage at the *Ase*I site made it possible to detect products of termination or replication past the dimer as a unique 2.3 kb band on an agarose gel. A 2.3 kb could also result from a subsequent round of replication of a mutagenic bypass product of the dimer, which would also render the *Ase*I site refractory to cleavage. When the dimer-containing vector was replicated in the HeLa extracts for various times, a 2.3 kb band was observed, along with the 1.6 and 0.7 kb bands

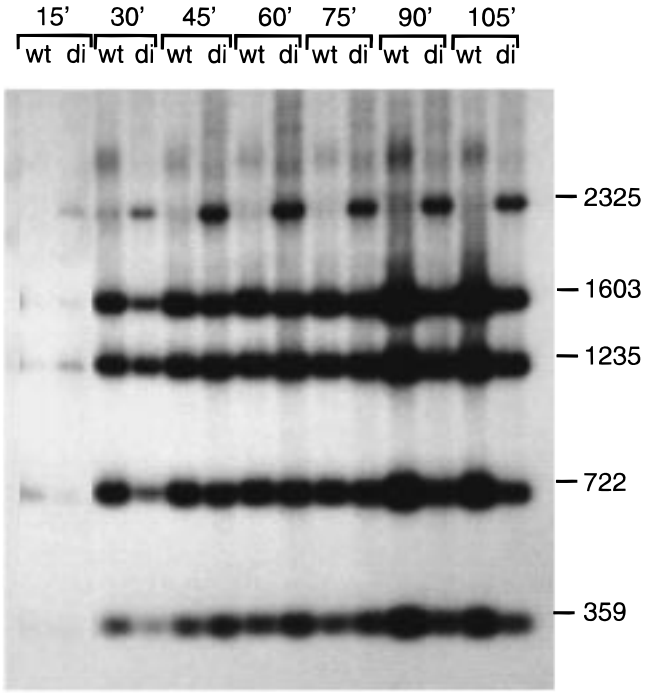

FIGURE 5: Time course of bypass and termination products. Autoradiogram of a 1% agarose electrophoresis gel of *dam* methylated wild-type (wt) and dimer-containing (di) pSVK3 that were replicated for the indicated times in minutes in the presence of [α-^{32}P]dATP, and then treated with *Ase*I. At short time points (15 and 30 min), the 2.3 kb band is primarily composed of leading strand dimer bypass and termination products, whereas the 1.6 and 0.7 kb bands represent the lagging strand synthesis products of the same section of DNA.

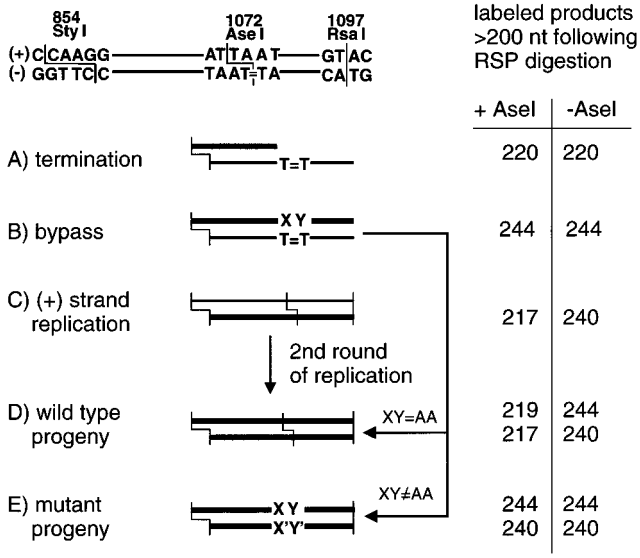

FIGURE 6: The restriction enzyme assay used to quantify the extent of termination and bypass at the dimer site (T=T). Unlabeled dimer-containing pSVK3 (thin lines) is incubated with human cell extract in the presence of [α-^{32}P]dNTP's and TAg to give radiolabeled products (thick lines). The replication products are then digested with *Rsa*I and *Pvu*II, and then split in two and digested with *Sty*I with or without *Ase*I. The termination product can be readily identified as a unique 220 nt band in digestions carried out in the absence of *Ase*I, and the bypass product of the dimer can be readily identified as a 244 nt band by its resistance to *Ase*I cleavage. Though there was no evidence for repair of the dimer, it is important to note that the bypass of a repaired dimer is *Ase*I cleavable and would lead to a 219 nt fragment.

expected for replication of the complementary strand (Figure 5). To assay for the amount of repair that was occurring during the replication reaction, the dimer-containing vector was incubated with the HeLa cell extracts under the replication conditions, except for the omission of TAg. No increase in the amount of cleavage at the *Ase*I site was observed (data not shown). The dimer-containing vector was also incubated under higher salt conditions which have been used to promote repair (*30*), also without any effect on *Ase*I cleavage at the dimer site. Our results are consistent with two previous studies that were unable to detect measurable levels of excision repair of vectors containing a single cis-syn dimer by whole cell extracts (*31*, *32*).

Denaturing Polyacrylamide Gel Assays of the Replication Products. The relative amounts of termination and bypass at the dimer site could be determined by autoradiography of denaturing polyacrylamide electrophoresis gels of replication reaction products following restriction digestion with combinations of enzymes (Figure 6). Cleavage with *Rsa*I and *Sty*I at the sites flanking the dimer site leads to a 244 nt (+) strand product and a 240 (−) strand product which are readily identified on a high-resolution gel. During one round of replication in the presence of [α-^{32}P]dNTP's, a radiolabeled 244 nt product would be primarily the result of leading strand replication of the (−) strand and a radiolabeled 240 nt band would be the result of lagging strand replication of the (+) strand. Termination of leading strand synthesis opposite the nucleotide prior to the dimer site would lead to a radiolabeled 220 nt band. To distinguish radiolabeled 244 nt products arising from bypass of the dimer from those arising from further replication of the initial progeny of the parental strands, the replication products were additionally treated with *Ase*I. Because the dimer inhibits cleavage of the *Ase*I site, the only radiolabeled 244 nt products are those that arise from bypass of the dimer, or from subsequent replication of the progeny of mutagenic bypass of the dimer (Figure 6). Radiolabeled 244 nt products arising from replication of the initial (+) strand progeny, as well as from a repaired dimer and progeny of nonmutagenic bypass of the dimer, would all be reduced in the presence of *Ase*I to 219 nt fragments. *Ase*I also reduces the radiolabeled 240 nt product of (+) strand replication to a 217 nt band. The replication products were also treated with *Pvu*II prior to treatment with *Ase*I to produce 231 and 235 nt fragments instead of 239 and 241 nt fragments that would otherwise result from *Ase*I(3910)-*Sty*I(231) cleavage and interfere with quantification of the 244 nt bypass band.

In the replication reactions of the dimer-containing vector treated with *Rsa*I, *Sty*I, *Pvu*II, and *Ase*I, a 219 nt fragment was only detected after 40 min (Figure 7a), and can only result from repair, which we found no independent evidence for, or from a second round or more of replication, which is consistent with the *Mbo*I digestion studies. Therefore the 244 nt band seen following incubation of the 20 min replication products with *Rsa*I, *Sty*I, and *Pvu*II, with or without *Ase*I, is the product of leading strand bypass of the dimer. When digested with *Rsa*I, *Sty*I, and *Pvu*II alone, the band that appears as a 220 nt fragment is the product of dimer-induced termination of leading strand synthesis and becomes more prominent at 40 min (Figure 7b). That termination occurred opposite the nucleotide to the 3′-side of the dimer could be established by comparing the termination band with those of a dideoxy sequencing ladders

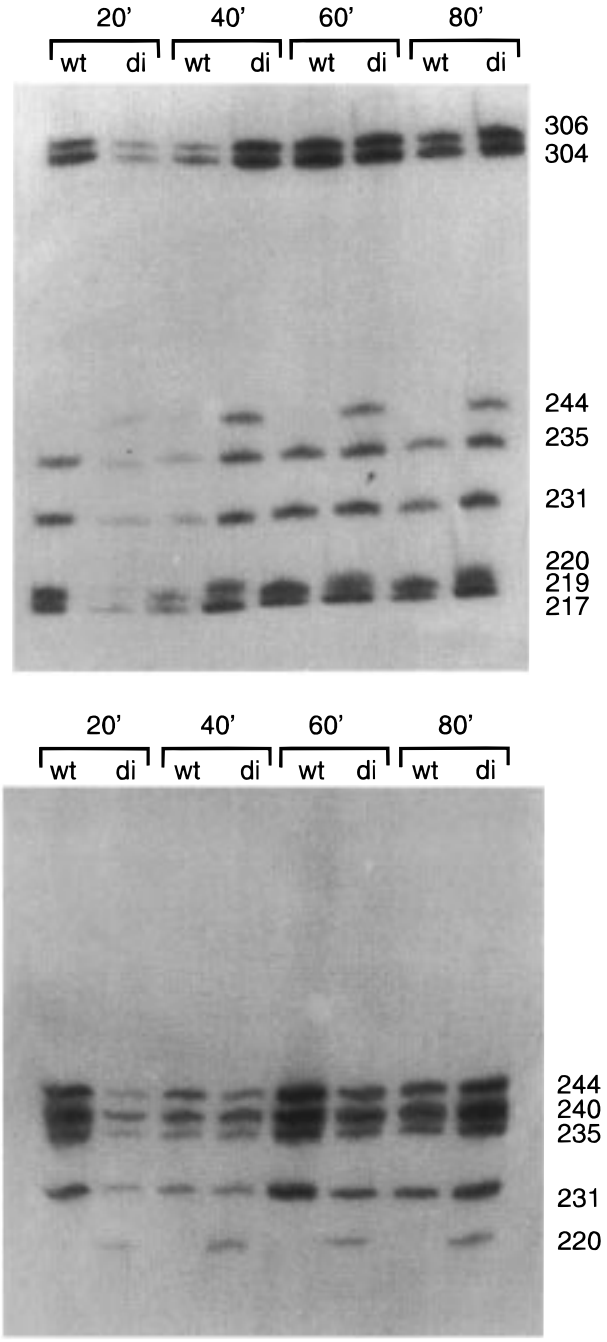

FIGURE 7: Time course of termination and replication past the dimer. Autoradiogram of an 8% denaturing polyacrylamide electrophoresis gel of the replication products of *dam* methylated wild-type (wt) and dimer-containing (di) pSVK3 for the indicated times in minutes that were digested with (a) *Rsa*I, *Sty*I, *Pvu*II, and *Ase*I (RSPA), and (b) with only *Rsa*I, *Sty*I and *Pvu*II (RSP). Replicative bypass of the dimer is indicated by the formation of a 244 nt band under RSPA digestion (gel a), and termination is indicated by the formation of a 220 nt band under RSP digestion (gel b). Multiple rounds of DNA replication are indicated by the formation of a 219 nt band under RSPA digestion (gel a).

generated using a primer corresponding to the 5′-terminus of the 244 nt fragment (Figure 8).

dNTP Concentration Studies. Under the standard conditions of the replication assay (25:100:100:100 [α-^{32}P]dATP:dCTP:dGTP:dTTP) the ratio of dimer bypass to termination varied from 0.8 to 1.3 at 20 min of replication for the majority of cell extract preparations, though in a few cases it was lower. In the limited number of cases studied,

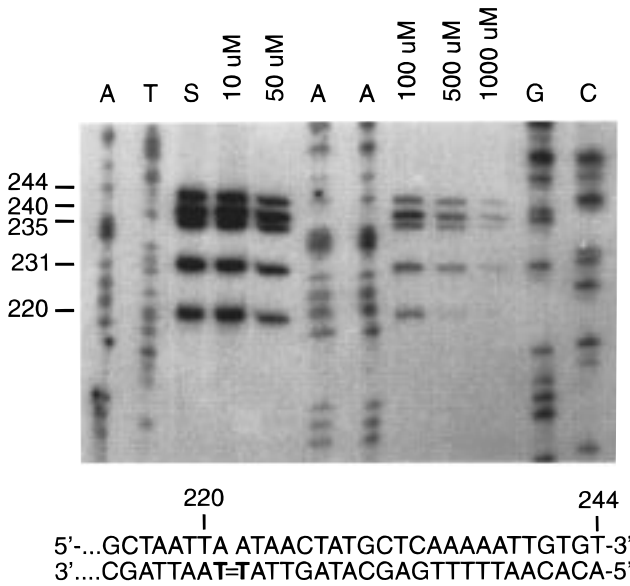

FIGURE 8: Effect of dNTP concentration on termination site and frequency opposite the dimer (T=T). Autoradiogram of an 8% denaturing polyacrylamide electrophoresis gel of the replication products of *dam* methylated dimer-containing pSVK3 that were carried out for 30 min with the indicated concentration of dNTPs and digested with *Rsa*I, *Sty*I, and *Pvu*II. The termination site was determined by alignment of the bands with bands in the dideoxy A, C, G, and T sequencing reaction lanes that were carried out with a 5′-[^{32}P]-end-labeled primer corresponding to the 5′-end of the *Sty*I 854, *Rsa*I 1097 fragment. Lane S corresponds to standard conditions of 100 μM dCTP, dGTP, and dTTP and 25μM dATP.

however, the effect of changing dNTP concentration was the same. When the replication was carried out for 20 min with dNTP concentrations ranging from 10 to 500 μM, the ratio of bypass to termination was found to increase from about 0.6 to 2.6 (Figure 9a). There was no obvious effect of dNTP concentration on the termination site, which always occurred opposite the A to the 3′-side of the dimer (Figure 8). The role of individual nucleotides in the bypass reaction was investigated by holding radiolabeled dGTP at 25 μM and raising the concentration of one, two, or three of the other dNTP's to 1 mM in various combinations. The ratio of bypass-to-termination ratio was greatest when three of the nucleotides were held at 1 mM, and least when only one nucleotide was held at 1 mM, with no clear preference for any single or pair of nucleotides (Figure 9b).

Replication by XPV Extracts. The ability of nontransformed primary XPV cell extracts to replicate the dimer-containing SV40 vector was also examined. The replication efficiency of these XPV extracts was found to be quite low compared to HeLa cell extracts (about 10 pmol dNMP/4 h vs 50 pmol/30 min) and was never sufficient for assaying the amount of dimer bypass. It was found, however, that recombinant human single-stranded DNA binding protein (rhRPA) greatly stimulated the replication efficiency of the XPV cell extracts (Figure 10). This experiment was originally motivated by the observation that replication in some HeLa extracts can be stimulated by rhRPA (M. Wold, personal communication). With our cytoplasmic HeLa cell extracts, stimulation of replication, as measured by an acid precipitation assay, depended on the extract used. In those HeLa cell extracts that were stimulated, addition of 200 ng rhRPA caused a 2−3-fold stimulation of the replication of

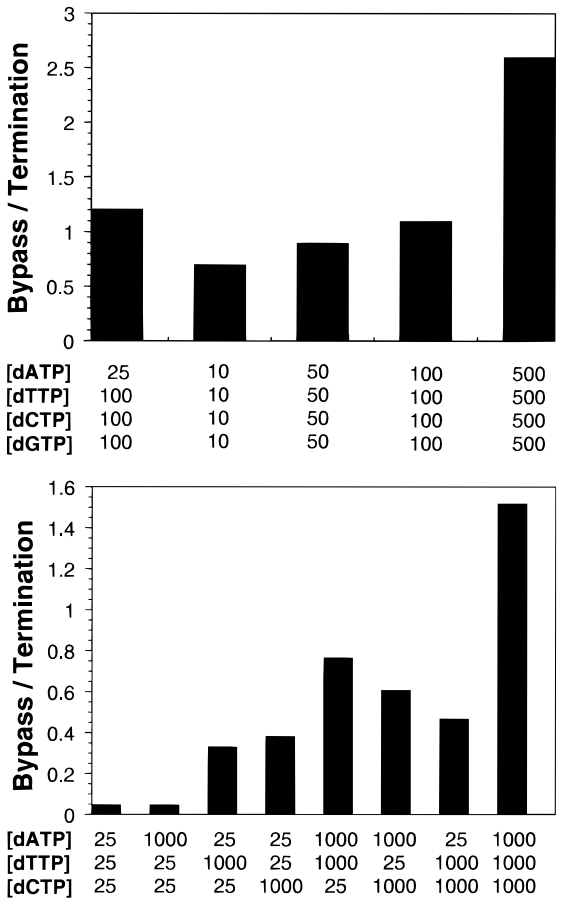

FIGURE 9: Quantification of the ratio of bypass to termination products of *dam* methylated dimer-containing pSVK3 as a function of dNTP concentration (μM). (a) Replication under the standard conditions (column 1) and in the presence of increasing dNTP concentration and [α-^{32}P]dATP, (b) replication under biased dNTP concentrations in the presence of 25 μM [α-^{32}P]dGTP for a different HeLa cell extract preparation.

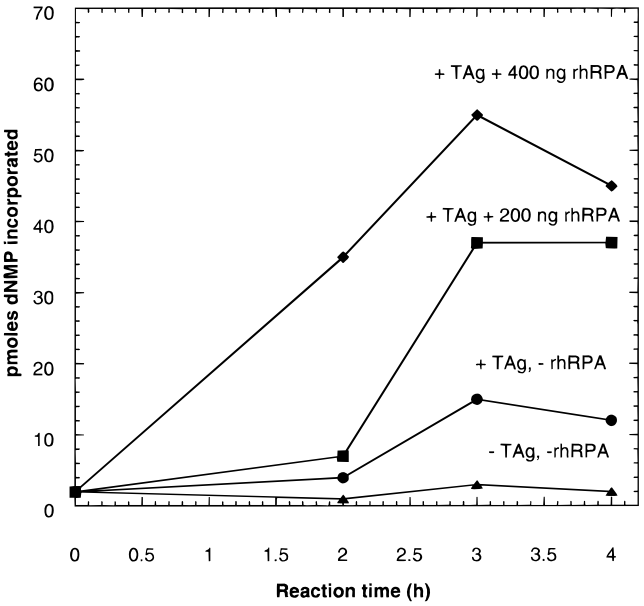

FIGURE 10: Kinetics of replication of *dam* methylated wild-type pSVK3 in the presence or absence of rhRPA and a typical cytoplasmic XPV extract showing replication activity. DNA replication reactions were run under standard conditions either with or without the addition of rhRPA or TAg. At the times indicated following incubation at 37 °C, an aliquot was withdrawn from each reaction and the extent of incorporation of radioactivity from [α-^{32}P]-dATP into trichloroacetic acid-precipitatable material was determined by liquid scintillation counting.

the wild-type plasmid (data not shown). On the other hand, cytoplasmic XPV extracts showing measurable replication activity were stimulated about 3−5-fold upon addition of rhRPA (Figure 10). Under these conditions, the XPV extracts were found to replicate past the dimer, though the amount of bypass appeared to be lower than that generally observed for the HeLa cell extracts (Figure 11). When directly compared on the same gel, DNA synthesis by XPV cell extracts appeared to terminate at the same position as did DNA synthesis by HeLa cell extracts.

DISCUSSION

In this paper we describe the replication products of the cis-syn thymine dimer-containing SV40 vector by cytoplasmic HeLa and XPV cell extracts. The goal of this work was to compare the results of a previous in vitro study of the DNA synthesis bypass of a cis-syn dimer with purified calf thymus polδ (*12*) with those of leading strand replication by wild-type and defective human replication systems.

Gel electrophoresis of HeLa cell replication products following treatment with restriction enzymes confirmed the formation of bypass and termination products and established that termination occurred opposite the A to the 3′-side of the dimer. These results are in agreement with what has been previously been reported (*7*). Termination was also found to occur almost exclusively one nucleotide prior to the 3′-T of the dimer with calf thymus polδ at 1−100 μM dNTPs in the absence of PCNA (*12*). In the presence of PCNA, however, increasing the dNTP concentration from 1 to 100 μM increased the amount of bypass and caused the termination site to advance opposite the 5′-T of the dimer. Though addition of PCNA has also been shown to greatly stimulate bypass of an abasic site analogue (53-fold), it did not appear to affect the site of termination, and only caused a minor increase in the bypass of aminofluorene (2.3-fold) and 8-oxo-dG (2.5-fold) adducts (*13*). When the dNTP concentration was increased from 10 μM to 500 μM in the cell-free HeLa extracts, the amount of cis-syn dimer bypass product was also seen to increase, but the termination site remained fixed opposite the A prior to the dimer (Figures 8 and 9a). Termination at the same position and frequency as observed with calf thymus polδ in the absence of PCNA suggests that the PCNA has become dissociated from polδ at the dimer site. In this regard, a previous study has indicated that the replication fork becomes uncoupled at the site of a cis-syn thymine dimer (*7*). Evidence that fork uncoupling may also be occurring in our system comes from analysis of the restriction fragments of the replication products. In the agarose gel assay, the sum of the 1.6 and 0.7 kb lagging strand fragments during the first round of replication is greater than the complementary 2.3 kb leading strand fragment composed of bypass and termination products (30 min lane, Figure 5). It is also possible, however, that the product terminating one prior to the dimer site results from some other process, such as exonucleolytic action by an occult nuclease or nuclease accessory factor that may be present in the crude extracts.

Another peculiar feature of the bypass reaction is that the greatest increase in the bypass-to-termination ratio was

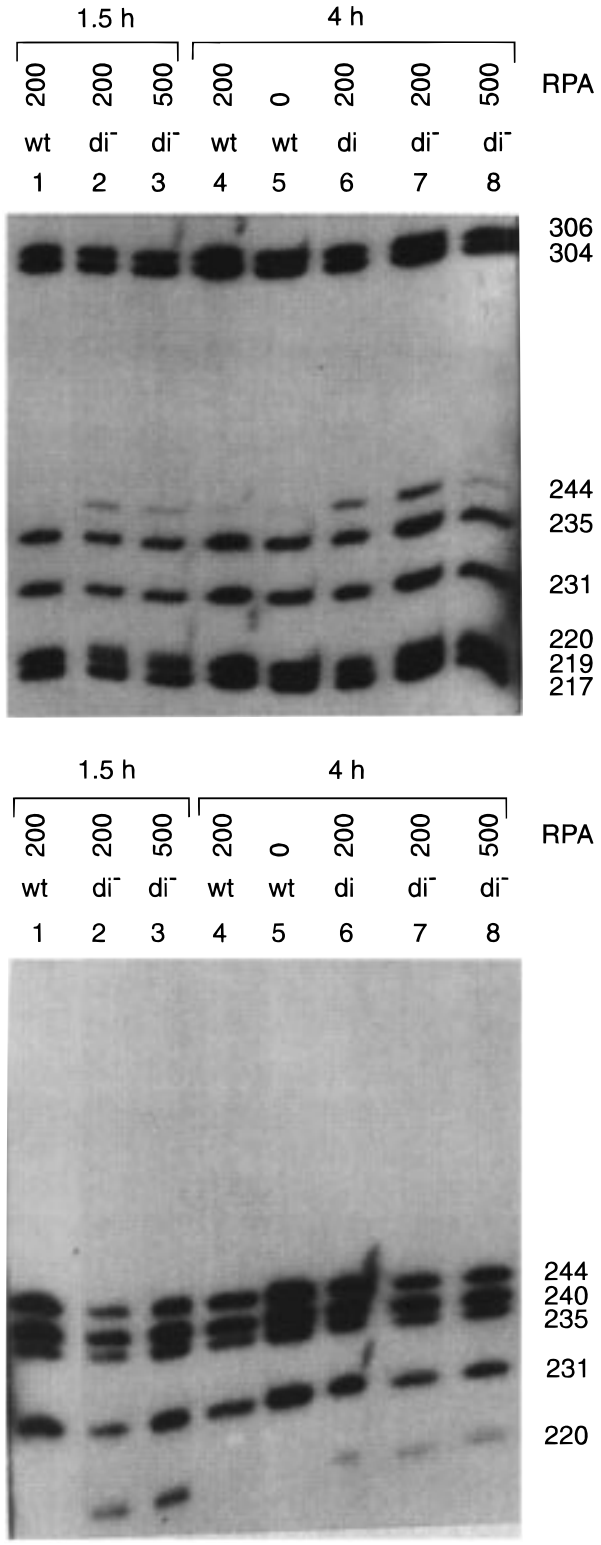

FIGURE 11: Autoradiogram of a polyacrylamide electrophoresis gel of the replication products of the *dam* methylated wild-type (wt) and methylated (di) or unmethylated (di⁻) dimer-containing pSVK3 by a cytoplasmic XPV extract in the presence or absence of the indicated amount of rhRPA in ng. (a) *Rsa*I, *Sty*I, *Pvu*II, and *Ase*I (RSPA) and (b) *Rsa*I, *Sty*I, and *Pvu*II (RSP) digestion products of 1.5 and 4 h replication reactions carried out under standard conditions as described in Materials and Methods. Replicative bypass of the dimer is indicated by the formation of a 244 nt band under RSPA digestion in gel a, and termination at the dimer is indicated by the formation of a 220 nt band under RSP digestion (gel b). Multiple rounds of replication are indicated by the presence of a 219 band under RSPA digestion (gel a).

observed for increasing the concentration of all dNTPs, yet only dATP and dTTP are expected to be immediately involved in the bypass reaction. The dimer is embedded in the center of a $(A/T)_9$ tract, and bypass has been shown by others to be nonmutagenic (*8*), and hence only As are involved in the synthesis opposite the dimer. One would therefore have expected that raising the concentration of dATP and dTTP alone would have been sufficient to effect the bypass rate, which was not observed (Figure 9b). This may suggest that bypass is not occurring by a simple translesion synthesis mechanism, but by some other mechanism.

With a few HeLa cell extracts, termination occurred more frequently than bypass during one round of replication, though in general the ratio of bypass to termination was approximately one under standard conditions. The variation in the ratio might be related to the replication efficiency of cytoplasmic extracts, and in particular how efficiently the replication machinery can be assembled. If, during the replisome assembly, the replication promoting proteins or factors have immediate access to polδ, which is responsible for leading strand synthesis in SV40 (*33*), the processivity of polδ may be enhanced and favor bypass.

Role of RPA in the Bypass of Dimers. In a transdimer synthesis mechanism, the dimer would transiently block DNA synthesis, and if fork uncoupling takes place, would generate a single-strand region downstream from the dimer site. Besides translesion synthesis, two other models, i.e., the recombinational transfer model in analogy to *E. coli* (*34*), and template switching (*35*) are proposed for the replication of damaged DNA. In all the bypass mechanisms, single-stranded DNA regions are produced during replication of the damaged DNA. Therefore, the single-strand binding protein RPA involved in DNA replication (*36*) might help to restore replication at the site of DNA damage when this process is completely or partially reduced due to artifacts of cell extract preparation, or genetic defects. In bypass-proficient extracts, additional RPA might not be involved at all or may play only a minor role in translesion synthesis because all factors or proteins responsible for replication of damaged DNA are sufficiently available.

When the replication products of primary untransformed XPV cytoplasmic extracts were assayed by restriction digestion and gel electrophoresis, we discovered that the XPV cytoplasmic extracts were also able to visibly bypass the cis-syn thymine dimer in the presence rhRPA, but less efficiently than HeLa extracts. This is in contrast to a recent report that cis-syn dimer bypass could not be detected in extracts of SV40 transformed XPV fibroblasts in the absence of added replication factors, whereas it could be detected in SV40 transformed normal and XPA fibroblasts (*9*). Although, for technical reasons, we could not carry out the bypass assay with the XPV extracts in the absence of rhRPA, these results open up the exciting possibility that XPV may be deficient in a biochemical pathway related to RPA.

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