Diffential Replication of a Single N-2-Acetylaminofluorene Lesion in the Leading or Lagging Strand DNA in a Human Cell Extract*

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DNA replication in eucaryotic cells is a complex process involving a variety of proteins that synthesize the leading and lagging strand in an asymmetric, coordinated manner. To investigate the effect of this asymmetry on the translesion synthesis of bulky lesions, we have constructed SV40 origin-containing plasmids with site-specific N-2-acetylaminofluorene adduct on either leading or lagging strand templates. These plasmids have been incubated with DNA replication-competent extracts made from human HeLa cells. Two-dimensional agarose gel electrophoresis analyses reveal a strong blockage of fork progression only when the N-2-acetylaminofluorene adduct is located on the leading strand template. Moreover, the analysis revealed that replication with HeLa cell extracts of SV40 origin-dependent plasmids functions in both directions from the origin with equal efficiency but, probably due to an important asynchrony at the formation of the two forks, proceeds unidirectionally for a large number of individual molecules. The validity of the in vitro replication approach to study the fidelity of both leading- and lagging strand synthesis is discussed with regard to these new data.

DNA lesions are usually repaired in an error-free manner, which eliminates most of the consequences of a DNA-damaging treatment. However, some DNA lesions may escape repair and can cause inhibition of DNA replication and transcription, alteration of gene expression, mutagenesis, or cell death. Hence, some mutations can be tolerated, but cells must replicate their full DNA before dividing. Because DNA replication is a complex process that functions in an asymmetric manner to coordinate synthesis of both leading and lagging strands (1), DNA adducts may have different potentials for blocking replication and therefore inducing mutagenesis, depending on which strand the lesion is located on. For example, in mammalian cells deficient in excision repair, mutations are supposed to be due to unrepai red lesions found only on one strand of the hprt gene (2–5), leading to the hypothesis that translesion synthesis of the leading strand is less accurate than that of the other strand. However, in cultured cells, the determination of the leading or lagging strand of the hprt gene has not yet been done.

Recent studies using in vitro SV40 origin-dependent replication with HeLa cell extracts have been made to determine the accuracy of leading or lagging strand synthesis (6–9). Translesion synthesis has been observed with a variety of types of DNA damage produced by chemical and physical agents, and mutation frequencies have been determined according to the position of the lesions. However, the conclusions that were drawn were based on at least two assumptions: first, that replication of SV40-based plasmids is bidirectional and synchronous from the SV40 origin under the conditions used for in vitro DNA replication; and second, that replication forks are similarly hindered by lesions on the leading or lagging strand template. We wished to re-examine the first point using a two-dimensional agarose gel electrophoresis technique, which allowed us to investigate the mode of replication of individual molecules (for a recent review, see Ref. 10). The second point has been addressed by studying the effect of the position of the adduct on replication fork progression. We have constructed plasmid molecules that contain a single N-2-acetylaminofluorene (AAF)1 in either the leading or lagging strand and used these DNAs as templates for replication in vitro in human HeLa cell extracts. AAF is a potent carcinogen that binds primarily at the C-8 position of guanine and has been shown to be a strong mutagen, leading essentially to 1- and 2-base deletions in Escherichia coli (11, 12). Minus two frameshift mutations have also been found after incubation of AAF-monomodified plasmid in HeLa cell extracts (7, 13). In this study, we have used the same set of SV40 origin-dependent DNAs to analyze the inhibition of the replication fork by lesions on either the leading or the lagging strand template. The usefulness of the in vitro SV40-based replication assay to study the fidelity of the two DNA strand synthesis is discussed on the basis of our findings.

EXPERIMENTAL PROCEDURES

Enzymes and Reagents—HeLa cytoplasmic extracts were prepared by the method of Li and Kelly (14). SV40 large tumor antigen (SV40 Tag) was obtained from Molecular Biology Resources (Milwaukee, WI). Creatine phosphate, creatine phosphokinase, and proteinase K were from Boehringer Mannheim. [32P]dCTP (1000–3000 Ci/mol) was from Amersham Corp. Ribonucleoside and deoxyribonucleoside triphosphates were from Pharmacia Biotech. Restriction endonucleases were from New England Biolabs.

Plasmids—Construction and description of substrates containing site-specific AAF adducts (pMKBNar and pMZBNar series) have been described previously (7, 15). SV40 DNA prepared according to the method of Hirt (16) followed by purification on a CsCl gradient was provided by Dr A. Gentil (Villejuif, France). Plasmid pVil was constructed by introducing the 2517-bp SpI DNA fragment of pACYC184, which contains the tetracyclin resistance gene, into the unique SpI site of pMKBNar (Fig. 1).

In Vitro Replication Assay—Replication reactions were carried out as described previously (17), with minor modifications. Reactions (50 μl) were performed in 30 mM HEPES (pH 8); 7 mM MgCl2; 4 mM ATP; 200 μM CTP, GTP, and UTP; 100 μM dATP, dGTP, dTTP and [32P]dCTP. DNA replication was assayed by polyacrylamide gel electrophoresis. The products were quantitated by phosphorimager and analyzed by densitometry.

1 The abbreviations used are: AAF, N-2-acetylaminofluorene; bp, base pair(s); RI, replication intermediate; SV40 Tag, SV40 large tumor antigen.
Replication Fork Inhibition by AAF Adducts

Unidirectional Like” Mode of Synthesis—
mode of replication and has already been used to detect pause

Two-dimensional Agarose Gel Electrophoresis Analysis—20 mg of carrier tRNA were added to the remainder of the reaction, and after a phenol-chloroform extraction, the mixture was filtered through a MicroSpin S-200 HR column (Pharmacia Biotech). After ethanol precipitation, DNA products were treated with StyI restriction enzyme for pMKBNar and pMZBNar (Fig. 1A), SflI endonuclease for SV40 DNA, and SflI or EcoRV endonuclease for pVil (Fig. 1B) before analysis by two-dimensional gel electrophoresis. The first-dimension electrophoresis was run at 0.6 V/cm for 14 h in a 0.4% agarose gel without ethidium bromide in 1× TBE buffer (90 mM Tris, 90 mM borate, 2 mM EDTA). The second-dimension electrophoresis was run in a cold room at 4 V/cm in a 1% agarose gel in 1× TBE buffer containing 0.3 mg of ethidium bromide (18). The dried gel was subjected to autoradiography.

Analysis of Replication Fork Movement from SV40 Origin—After in vitro replication, DNA products were purified and digested with three restriction endonucleases: BglI, AflIII, and Bsu36I. The fragments were separated by 5% nondenaturing polyacrylamide gel electrophoresis. The gel was then dried and exposed to a phosphor screen. The amount of labeled dCMP incorporated was quantified using ImageQuant software (Molecular Dynamics). DNA synthesis was normalized by dividing the gel intensities by the number of cytosine residues in each fragment to account for the relative incorporation of radioactive dCMP into each fragment.

RESULTS

To detect replication fork blockage by a unique acetylaminofluorene adduct, we analyzed the replication intermediates (RIs) by two-dimensional agarose gel electrophoresis. This technique provides useful qualitative information about the mode of replication and has already been used to detect pause sites in DNA synthesis (19–21).

In Vitro Replication of Unmodified Plasmid Proceeds by a "Unidirectional Like" Mode of Synthesis—Unmodified monomer plasmid DNAs were incubated in the presence of HeLa cytoplasmic extract at 37°C as described in “Experimental Procedures.” DNA synthesis was SV40 Tag-dependent, and no incorporation could be detected when plasmid without a SV40 origin was used. About 25 pmol of dCMP were incorporated after a 60-min incubation with either of the two pMKBNar or pMZBNar plasmids (47 and 114 pmol of dCMP incorporated for 2 and 4 h of incubation, respectively). Reaction products were digested with StyI restriction enzyme, which cuts once at the origin (Fig. 1A), and then analyzed by two-dimensional agarose gel electrophoresis (Fig. 2).

For both plasmids, a simple Y arc was detected. This pattern is characteristic of DNA fragments with a unique replication fork coming from one extremity. A double Y profile that would correspond to RIs with two replication forks coming form both ends was not clearly observed. However, a very light and diffuse triangular region was detected. This indicates the convergence of opposing replication forks at multiple sites throughout some molecules. This analysis suggested that replication proceeds mostly unidirectionally on individual molecules. The result was unexpected because previous studies suggested that SV40 origin containing plasmids could replicate by a bidirectional mode (14, 22, 23).

Plasmids used in this study possessed a minimal SV40 origin and were relatively small (2.7 kb). To see whether the unidirectional mode of replication was due to the initiation of synthesis at this minimal origin, we performed the same experiment with DNA directly isolated from the SV40 virus. In parallel, to follow the effect of plasmid size, we constructed a new plasmid, pVil, in which the 2517-bp SspI DNA fragment of pACYC184, containing the tetracyclin resistance gene, was cloned into the unique SspI site of pMKBNar (Fig. 1). In this way, we increased the size of the plasmid from 2.7 to 5.3 kb (size identical to that of wild type SV40 DNA) with the same minimal origin of replication (Fig. 1B). The replication products derived from SV40 DNA virus and pVil plasmid were digested with the SfiI enzyme, which cuts once at the origin, and analyzed by two-dimensional gel electrophoresis.

We never succeeded in getting a complete digestion with SfiI endonuclease, even with large amounts of enzyme and prolonged incubation times. SfiI endonuclease cleaves DNA by a mechanism that differs from other restriction enzymes and is known to cut inefficiently substrates with only one SfiI site (24, 25).
two-dimensional analysis gave a clear bubble to double Y transition (Fig. 3B). Such a pattern demonstrated that in this assay, replication occurred mostly via only one fork (26). In addition, an unexpected simple Y arc was detected. Because more than 94% of the input pVil plasmid is monomer, it seems unlikely that this simple Y arc resulted from replicated dimers (27). These simple Y molecules were probably due to some initiation started randomly through the DNA. However, this random initiation appeared only with plasmid having a SV40 origin and requires the SV40 origin and requires the SV40 origin and requires the SV40 origin.

The existence of double Y-shaped molecules detected for both plasmids cleaved with SfiI endonuclease (see “Experimental Procedures”) proves that all components were present in the cytoplasmic Hela cell extract to perform bidirectional replication. The minimal SV40 origin in pVil plasmid, and consequently in pMKBNar and pMZBNar plasmids, behave in the same manner as the complete wild type SV40 origin of replication.

Although pVil and pMKBNar possess the same SV40 origin, molecules with two symmetrical forks arriving on opposite directions could be seen only with pVil. One possible explanation, for the small plasmid, is that one fork becomes functional and replicates the whole plasmid before the other fork has time to become operative. Due to the larger size of the plasmid pVil (which is identical to that of wild type SV40 DNA), sufficient time elapses before the completion of replication of the first fork to allow the second fork to start synthesis.

DNA Replication Can Start in Both Directions—Two-dimensional gel analysis showed that replication proceeds unidirectionally in about half of the molecules. However, because SV40 origin-dependent synthesis can start from two opposite directions, the data do not exclude the possibility that 50% of the molecules replicate in one direction and 50% in the other. To examine this possibility, plasmid pVil was labeled for short periods in an in vitro assay and then digested with three restriction enzymes, BstU1, BglII, and AluI. The fragments were resolved on 5% non-denaturing polyacrylamide gels. DNA synthesis was quantified by determining the corrected labeling of each fragment (Fig. 4).

After 15 min of incubation, only fragments D, E, and F, directly on each side of the origin, became labeled. After 20 min of incubation, the amount of label in fragments close to the origin increased, and some incorporation was detected in fragments B and C. Finally, labeling of fragment A, which is opposite the origin, was detected at the 30-min time point. At each time point, the incorporation of labeled DCMP followed a bell curve centered around the origin. These data are in agreement with those obtained with SV40 DNA (22, 23). They demonstrate that pVil DNA replication started from the origin sequence, and at this level of analysis, replication appeared to proceed in both directions from the origin with equal efficiency.

Selective Blockage of Fork Progression by a Unique AAF Adduct Located on the Leading Strand—To study the inhibition of fork progression by a bulky adduct, we used site-specific AAF-modified plasmids pMKBNar and pMZBNar. These vectors have been described elsewhere (7). Briefly, the two plasmids differ only in the orientation of the HaeII restriction site.

FIG. 2. Two-dimensional agarose gel analysis of unmodified plasmids. 20 ng of pMKBNar or pMZBNar were incubated for 1 h in presence of HeLa cytoplasmic extracts (see “Experimental Procedures”). After digestion with Styl restriction enzyme, which cuts once at the origin, labeled DNA products were analyzed on two-dimensional agarose gels. A, autoradiography reveals an arc representing Y-shaped molecules. A very faint triangular pattern on the top of the Y arc indicates the presence of double Y-shaped molecules with termination of replication at many sites near one end of the segment. B, diagram showing the theoretical shape of the different DNA molecules found after replication of unmodified pMKBNar and pMZBNar. In represents the size of monomer linear plasmids, and 2n indicates linear molecules that are almost replicated before separation in two monomers.

25). After digestion, two species coexisted in about the same proportion, linear DNA and nicked circular DNA (data not shown). But because no other enzyme could be used for cutting at a unique site in the replication origin, we decided to analyze the uncomplete restriction reaction product on two-dimensional gel (Fig. 3A).

For both plasmids digested with Styl endonuclease, a simple Y arc and a double Y arc were present. This indicates that some molecules replicated with only one replication fork, starting at the origin, and some replicated via a bidirectional mode. Another large spot, detected just on the top of the linear monomer, is attributed to nicked circular monomer. From this spot, a new arc starts, probably representing RIs with only one of the two replicated daughter strands cleaved by Styl enzyme.

To confirm the presence of unidirectionally synthesized molecules, we performed an additional experiment, in which replication products derived from pVil plasmid were digested with EcoRV, which cuts once at the opposite of the origin. The
fragment containing the entire lacZ α complementing gene (Figs. 1 and 5). When modified with AAF on the third guanine (Gβ) of the Nar1 site (5'-G1G2CG3CC), the pMKBNar substrate contains the adduct on the leading strand template with respect to the closest replication fork emanating from the SV40 origin. With pMZBNar DNA, the adduct is located on the lagging strand template in the same sequence context. The distance from SV40 origin to the AAF adduct is 523 nucleotides for pMKBNar and 588 nucleotides for pMZBNar, representing 19 and 21%, respectively, of the total size of the plasmid.

Undamaged and site-specific modified substrates were replicated for 1 h in the HeLa cell extract. With AAF DNA, synthesis is about 30% less efficient than with the unmodified substrates. Following extraction and precipitation (see "Experimental Procedures"), samples were subjected to two-dimensional electrophoresis (Fig. 5). For both unmodified DNAs, as described before, a smooth simple Y arc was detected (Fig. 2).

With AAF-modified pMKBNar, an accumulation of RIs at a specific site was visible (Fig. 5B). These molecules migrate like linear molecules of approximately 3350 bp. This corresponds to RIs of Y forms with two short arms of ~560 bp in length. The distance between the StyI site and the AAF adduct is 572 nucleotides. Therefore, these RIs represent molecules with replication fork 1 blocked at the unique AAF lesion. This fork “sees” the adduct on the leading strand. In addition, some double Y forms can be detected, starting from RIs with the inhibited fork 1. They represent molecules with replication fork 2 coming from the opposite direction. Indeed, because fork 1 is stalled at the lesion, fork 2 has time to start and achieve replication of the molecule. The signal of the double Ys has a hook at the end, which is exactly what it is expected for a unique class of molecules with two forks that meet at an asymmetric location (26). This is an additional proof for the asynchrony in the initiation of the two replication forks.

With AAF-modified pMZBNar, a strong spot corresponding to accumulated RIs of ~5000 bp lies on the simple Y arc (Fig. 5D). These Y-shaped molecules possess two arms of ~2210 bp each. This is exactly the distance that fork 2 has to move before encountering the lesion. For this fork 2, the adduct is on the leading strand. The presence of double Y forms starting from this spot indicates that in some molecules, when fork 2 is stalled at the lesion, fork 1 becomes functional, as shown in Fig. 5D. Using this site-specific modified vector, no inhibition of fork 1 can be detected. Taken together, these data mean that replication forks are blocked only by adducts located on the leading strand template, and in this case, the other replication fork can still progress toward the first fork with some retardation.

**DISCUSSION**

Previous work has suggested that only lesions located on the leading strand are capable of inhibiting the progression of replication forks, but no direct evidence has been shown yet. The purpose of this study was to test this hypothesis using DNA plasmids modified with a single site-specific AAF adduct on either the leading or lagging strand template. In this work, several unexpected results were found concerning the mode of replication of SV40 origin-dependent DNA after incubation with HeLa cell extract.

**In Vitro Replication of Unmodified DNA in a HeLa Cell Extract**—The technique of two-dimensional agarose gel electrophoresis provides detailed information about the mechanism of replication (10). StyI-digested replication intermediates that result from in vitro synthesis of pMKBNar and pMZBNar in the presence of HeLa cell extract migrate along a simple Y arc,
indicating that plasmids are replicated mostly by only one replication fork. A very faint triangular pattern indicated the presence of plasmids with two replication forks moving in the opposite direction. We decided to test whether this “unidirectional” mode of replication at the origin was due to the use of a minimal SV40 origin. SV40 DNA was replicated in vitro with HeLa cell extract, and labeled products were analyzed by two-dimensional agarose gel electrophoresis. As with previous plasmids, a simple Y arc was detected, meaning that a lot of SV40 molecules are replicated by only one replication fork. However, a very clear double Y line is now detectable, corresponding to replication intermediates with two forks. Some molecules were therefore replicated bidirectionally. These data are consistent with those obtained by Dhar and Schildkraut (20) using the same system; they also found the presence of highly asymmetric molecules. However, the proportion of molecules with two replication forks seems to be higher in their study. Reasons for this discrepancy are not clear; some difference in quality and efficiency of the extract as a function of cell culture conditions or mode of extraction may be important factors. In our case, we tested two different HeLa extract preparations and obtained exactly the same results.

Two parameters differ between SV40 DNA molecules and pMKBNar or pMZBNar plasmids: the sizes (5243 bp and 2782 bp, respectively) and the origin, which is complete in SV40 DNA and “minimal” in pMKBNar. To examine whether RIs with two replication forks diverging from the origin can be obtained with the minimal origin, we constructed a new 5303-bp plasmid, pVil, which contains the whole pMKBNar plus an extra DNA sequence not related to SV40. The pattern obtained with pVil after replication and SfiI digestion on two-dimensional gel analysis was exactly the same as the one observed with SV40 DNA. Therefore, whatever the origin used in the in vitro replication assay, molecules with bidirectional synthesis are present. The size of the DNA seems to be crucial to detect RIs with two forks.

Because two forks can assemble at the SV40 origin and a large proportion of the input plasmid is replicated by only one fork, we examined which one is functional. One-dimensional agarose gel electrophoresis analysis revealed that about 50% of molecules are duplicated in one direction and 50% in the other. In other words, analysis made at the population level shows that replication proceeds in both direction from the origin, but at the individual level, a large proportion of molecules are replicated in an unidirectional mode.

This idea was first proposed and rejected by Stillman and Gluzman (22). The rejection was based on the data of Li and Kelly (14), who showed electron microscope pictures of replicative intermediates of SV40 DNA incubated in the presence of COS-1 cell extract. In this study, newly replicated viral DNA with two forks going in opposite directions were detected. They also found some RIs presenting fork asymmetrically distributed compared with the origin and interpreted them as asynchrony in fork progression. It is possible that some factors necessary for synchrony of fork movement or initiation are present in COS extract and in limiting amounts in HeLa cell extract. Indeed, COS cells are green monkey kidney cells, which are the natural host of the SV40 virus, expressing a high level of SV40 Tag, whereas HeLa cells are semipermissive for SV40 replication. A second point should be stressed at this time. The two-dimensional electrophoresis analysis takes into account all the replicated DNA, but electron microscopy focuses only on small numbers of individual molecules. Our data are not formally in disagreement with those of Li and Kelly (14). SV40 and pVil synthesis revealed the presence of molecules with two forks diverging symmetrically from the origin, but a large proportion of them present such high asynchrony that they seem to be replicated unidirectionally.

Strong Inhibition of Replication Fork Progression When AAF Is Located on the Leading Strand Template—Previous studies have shown a strong inhibition of the replication fork by different DNA lesions, such as UV (28–31) or AAF lesions (32).
Given the asymmetry of the DNA replication process, a DNA adduct may have different capacities for blocking replication depending on whether the adduct is located on the leading or lagging strand template. The mechanism by which lesions inhibit eucaryotic DNA replication are still under investigation. One model suggests that lesions in the leading strand template block replication fork progression, whereas lesions in the template for discontinuous synthesis block only completion of an Okazaki fragment, without hindering the fork movement (31–33). We directly tested this model using AAF-monomodified plasmids. In a reaction containing either a single strand template, the same result was obtained with plasmids AAF-monomodified plasmids were on the leading strand relative to the proximal fork, but on the leading strand template for fork 2. D, accumulated RIs are now found in molecules with higher molecular weights. Determination of the size reveals that in these molecules, the distal fork (fork 2) is stalled at the AAF lesion. No inhibition of fork 1 was detected.

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