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Benzo[*a*]pyrene diol epoxide I binds to DNA at replication forks

(transformation/adduct/antibody/electron microscopy)

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ABSTRACT **The distribution of lesions in DNA caused by (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (B[*a*]P diol epoxide-I) was studied in synchronized C3H/10T½ cells treated in S phase. Sites of carcinogen modification of DNA were identified by polyclonal rabbit antibodies elicited against DNA modified with B[*a*]P diol epoxide-I *in vitro*. This antigenic DNA contained *trans*-(7*R*)-N^2-[10-(7β,8α,9α-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene)-yl]-deoxyguanosine; other adducts were not detected by liquid chromatography. In this study, DNA replication forks with antibodies bound to B[*a*]P diol epoxide-I adducts were detected by electron microscopy. The frequency of replication forks containing carcinogen adducts associated with the fork junction was found to be 8-fold higher than expected for an average distribution. The proportion of replication forks that were apparently blocked at the site of the DNA damage increased when replication was allowed to occur after carcinogen exposure. These results support the conclusions that the fork junction is particularly vulnerable to adduction by B[*a*]P diol epoxide-I and that B[*a*]P diol epoxide-I adducts block the displacement of replication forks during DNA synthesis in intact cells.**

Proliferation of cells after exposure to chemical carcinogens is important to the initiation of malignant transformation. In studies of carcinogenesis in mouse skin, increased susceptibility to tumor formation correlated with increased DNA synthesis after carcinogen treatment (1, 2). In partially hepatectomized rats, the maximal sensitivity to tumor formation in the liver was observed when carcinogen treatments occurred during the S phase of proliferating hepatocytes (3, 4). Transformation of cells in culture is also dependent on cell proliferation and DNA replication immediately following the carcinogen treatment (5). The maximal sensitivity to transformation of mouse fibroblasts was observed when cells were exposed to the carcinogenic agent during the early portion of the S phase (6, 7). To investigate this sensitivity, we studied the effect of exposure to (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo-[*a*]pyrene (B[*a*]P diol epoxide-I) on C3H/10T½ cells actively synthesizing DNA.

B[*a*]P diol epoxide-I, the ultimate carcinogenic metabolite of benzo[*a*]pyrene (8), modifies nucleic acids by linkage of the exocyclic 2-amino group of guanine and the C-10 position of the B[*a*]P diol epoxide-I moiety (9). The mechanisms by which B[*a*]P diol epoxide-I acts to induce malignant transformation are unclear. Evidence has been reported for the occurrence of specific mutations (10, 11) and cytogenetic changes, such as sister chromatid exchange (12–14), in the DNA of cells exposed to B[*a*]P diol epoxide-I. In subcellular systems *in vitro* DNA synthesis is blocked at each DNA adduct encountered by the polymerase (15–17). In eukaryotic systems DNA adducts block DNA chain elongation and cause gaps to be formed in nascent DNA (13, 18–21). However, in most nonlethal cases, DNA synthesis resumes with time, and gaps, as well as B[*a*]P diol epoxide-I-DNA adducts, decrease or disappear.

In the past, information concerning the effects of B[*a*]P diol epoxide-I exposure on DNA replication was obtained primarily from studies of the size distribution of nascent DNA molecules by using alkaline sucrose gradient sedimentation or alkaline elution (13, 18–21). Such studies depended on detection of changes in large populations of DNA molecules, and some correlated the number of B[*a*]P diol epoxide-I adducts present in DNA with the reduction in either the size of nascent DNA fragments (18) or the rate of DNA synthesis (21). Correlations of this type permit only indirect inferences to be drawn concerning the effects of B[*a*]P diol epoxide-I adducts on DNA synthesis. In the present study a different approach was used that allows a direct analysis of the effect of B[*a*]P diol epoxide-I adducts on DNA replication. The basic methodology uses antibodies with high affinity for B[*a*]P diol epoxide-I adducts attached to the exocyclic nitrogen of deoxyguanosine in DNA and uses electron microscopy to visualize and quantitate antibodies bound to B[*a*]P diol epoxide-I DNA adducts (22). We now report the results of investigations of the effects of B[*a*]P diol epoxide-I on 10T½ cells treated in mid-S phase. We used our quantitative methodology to investigate the actual distribution of carcinogen-DNA adducts in proximity to DNA replication forks.

MATERIALS AND METHODS

Maintenance of Stock Cell Cultures. Mouse C3H/10T½ clone 8 (23) cells (obtained from C. Boreiko, Chemical Industries Institute of Toxicology, Research Triangle Park, NC) were maintained in logarithmic growth and were used between passages 10 and 16. Stock cultures were grown in basal medium Eagle containing 10% (vol/vol) heat-inactivated fetal bovine serum, $NaHCO_3$ at 2.2 g/liter, and Hepes at 6 g/liter (pH 7.2). Cells were incubated in a humidified atmosphere of 5% CO_2/95% air at 37°C. Stock cultures were maintained without the use of antibiotics and were routinely shown to be free of mycoplasma contamination (24).

Synchronization of Cell Populations. Cells were synchronized by growth arrest at confluence followed by replating at low density (6). Logarithmically growing 10T½ cells were seeded at 300,000 cells per 100-mm dish. Cells were fed on

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Abbreviation: B[*a*]P diol epoxide-I, (±)-7β,8α-dihydroxy-9α, 10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene.

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day 4 and allowed to grow to confluence until day 8. Cells were trypsinized and reseeded at 300,000 cells per 60-mm plate. Experiments were performed 20–24 hr later, when cells were in mid-S phase.

Kinetics of Inhibition of DNA Replication Following B[*a*]P Diol Epoxide-I Exposure. Synchronized cells were exposed to 1.7–3.3 μM B[*a*]P diol epoxide-I in Hanks' balanced salt solution containing Ca^{2+} and Mg^{2+} for 10 min. Cultures were fed with complete medium and later incubated with [^{3}H]thymidine at 45 μCi/ml (60 Ci/mmol; 1 Ci = 37 GBq; Schwarz/Mann) during a 5-min interval at various times after the end of carcinogen treatment. For each of the three treatment groups (control, 1.7 μM B[*a*]P diol epoxide-I, and 3.3 μM B[*a*]P diol epoxide-I), four dishes were treated per time point. Medium was removed after labeling, and cell cultures were washed twice with ice-cold saline, five times with 5% (wt/vol) trichloroacetic acid, and three times with 95% (vol/vol) ethanol. Dishes were incubated overnight at 37°C with 0.3 M NaOH. Absorbances at 260 nm were measured for the NaOH-solubilized cellular material. Aliquots of NaOH-solubilized material were neutralized with HCl, and radioactivity was determined (20, 21).

Analysis of Replicating DNA Modified by B[*a*]P Diol Epoxide-I. Cells in mid-S phase were exposed to 3.3 μM B[*a*]P diol epoxide-I under two specific conditions. In group I, cultures were incubated at 37°C prior to B[*a*]P diol epoxide-I treatment with complete medium containing aphidicolin at 2.0 μg/ml (Natural Products Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD). After 30 min, the medium was removed, cell cultures were rinsed twice with 5 ml of saline that contained aphidicolin at 2.0 μg/ml, and 20-μl aliquots of tetrahydrofuran-containing B[*a*]P diol epoxide-I were added to an additional 5 ml of saline with aphidicolin. Cultures were exposed to B[*a*]P diol epoxide-I for 10 min at room temperature, saline containing B[*a*]P diol epoxide-I was removed, and complete medium with aphidicolin was added to each dish. Cultures were incubated for 60 min at 37°C. In group II cells were rinsed twice with saline, and treated with B[*a*]P diol epoxide-I as described above, but in the absence of aphidicolin. Once B[*a*]P diol epoxide-I was removed, 5 ml of complete medium was added to each dish, and the cultures were incubated for 70 min at 37°C. At the end of the incubations at 37°C, DNA was purified from cell cultures from both group I and group II and later digested with the restriction endonuclease *Pvu* II (Bethesda Research Laboratories), as described (22). DNA solutions in 10 mM Tris·HCl/100 mM NaCl/1 mM EDTA, pH 7.5, were divided into aliquots, and those that were not used immediately were stored at −20°C.

Analysis by Electron Microscopy of Antibodies Bound to B[*a*]P Diol Epoxide-I Adducts. DNA samples from group I and group II were prepared for visualization of antibodies as described (22). By using levels of modification as determined by ELISA, duplicate 20-μg samples of DNA were incubated with ≈25 times the estimated minimal amount of B[*a*]P diol epoxide-I-DNA antiserum necessary to react fully with those adducts present. After 9 hr at 37°C, an equal volume of papain in 0.20 M potassium phosphate, pH 7.0/0.02 M L-cystine hydrochloride/0.004 M EDTA was added to the DNA–antibody reaction mixture. The papain solution was prepared such that 1 mg of papain would be added to the final solution for each 100 mg of protein already present in the DNA–antibody reaction mixture. After 15 hr at 37°C, the volume of the samples was reduced, and B[*a*]P diol epoxide-I-DNA–Fab complexes were separated from the digestion mixture by column exclusion chromatography. The complexes then were incubated with amounts of ferritin-conjugated univalent goat anti-rabbit F(ab')$_2$ fragments that were ≈10 times the amount necessary to react with all of the rabbit B[*a*]P diol epoxide-I-DNA–Fab complexes present. After incubation for 15 hr at 37°C, sample volumes were again reduced, and DNA–antibody complexes were purified and prepared for analysis with the electron microscope. For analysis of the proximity of sites of carcinogen modification to the junction of DNA replication forks, DNA molecules were examined with the electron microscope in a systematic manner, so that each field was analyzed only once. When DNA molecules that resembled the "Y" conformation of DNA replication fork structures were visualized, they were examined at various magnifications. If these DNA molecules resembled replication fork structures, they were recorded as such. If a replication fork structure had an electron-dense ferritin molecule associated with it, a photograph was made of the DNA molecule at a magnification of ×50,000. In addition, after photographing a DNA molecule recorded as a replication fork with antibody, the next apparent DNA replication fork structure without antibody was photographed at the same magnification to create a parallel population of fork structures without antibodies. Subsequently, electron micrographs were closely inspected to confirm that the photographed DNA molecules resembled acceptable DNA replication fork structures. To be acceptable as an apparent fork structure, DNA molecules had to have three clearly discernable branches originating from one apparent junction. In addition, two of the three branches had to be of approximately the same length because the restriction endonuclease should cleave the two newly replicated daughter molecules at identical recognition sites. The fact that methylation at the internal cytosine of the *Pvu* II recognition sequence could prevent cutting at the methylated site should have no effect on our analyses. The frequency of methylated cytosines is small and both daughter DNA molecules should have the same methylation pattern (fully methylated, hemimethylated, or nonmethylated) at each individual site. Lengths of the strands of DNA fork structures were determined by tracing, as described (22).

RESULTS

Inhibition of DNA Synthesis. Synchronized populations of 10T½ cells in mid-S phase were exposed to either of two concentrations of B[*a*]P diol epoxide-I. The kinetics of inhibition of DNA replication were measured, and the results are shown in Fig. 1. For cell cultures exposed to 1.7 μM B[*a*]P diol epoxide-I, there was a rapid decline in the incorporation of [^{3}H]thymidine during the first 15 min after treatment. This changed to a more gradual decline, and after 90 min the rate of DNA synthesis was 32% of that observed in control cultures. For cell cultures exposed to 3.3 μM B[*a*]P diol epoxide-I, there was an even more dramatic and immediate reduction in the incorporation of [^{3}H]thymidine. After 45 min the rate of DNA synthesis in B[*a*]P diol epoxide-I-treated cultures leveled off at 13% of the value for untreated cell cultures.

Visualization of Antibodies Bound to Sites of Carcinogen Modification of DNA. Synchronized cell cultures were exposed for 10 min to 3.3 μM B[*a*]P diol epoxide-I in mid-S phase and incubated for 60–70 min in medium free of carcinogen. Cell cultures in group I were maintained all the time in the presence of aphidicolin at 2.0 μg/ml, a potent inhibitor of DNA polymerase α (25). Cell cultures of group II were exposed to carcinogen and incubated in the absence of aphidicolin. DNA was isolated and was prepared for visualization of antibody-bound sites of carcinogen modification. DNA replication forks were scored for the presence and the position of ferritin-labeled antibodies (Fig. 2). In Table 1 we listed both the number of total fork structures tagged with a ferritin-labeled antibody anywhere in the DNA molecule and a subgroup of these fork structures in which the ferritin-labeled antibody was localized to the junction region of the replication forks [i.e., within 80 base pairs (bp) of the tran-

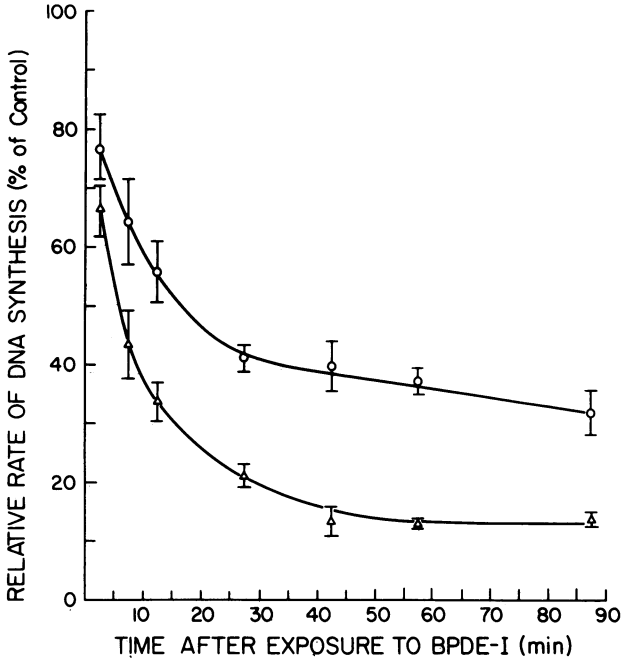

FIG. 1. Relative rate of DNA synthesis at various times after exposure to B[*a*]P diol epoxide-I (BPDE-I). Synchronized populations of C3H/10T½ Cl8 cells were exposed in mid-S phase to 0, 1.7 (○), or 3.3 (△) μM B[*a*]P diol epoxide-I for 10 min. At various times after B[*a*]P diol epoxide-I exposure, cells were pulsed for 5 min with [^{3}H]thymidine. Rates of DNA synthesis, as indicated by amount of radioactivity per A_{260} unit, were expressed as percent of the amount of radioactivity per A_{260} unit determined for parallel cultures treated with the carcinogen solvent. Four dishes were used per data point.

sition point between nonreplicated and daughter branches of the fork structures]. We observed that when replication was allowed to proceed after the exposure to B[*a*]P diol epoxide-I (group II), 27% of the observed fork structures had antibodies associated with them, as compared to 20% in group I (replication inhibited by aphidicolin). More specifically, DNA replication forks that had ferritin-labeled antibodies located at the junction region of the Y structures (Fig. 2) represented 8% of the total number of forks in group I and 13% in group II. The statistical significance of the difference between group I and group II in the number of DNA replication forks with ferritin-antibodies at the junction region was evaluated by a χ^2 test. The result was an 80% probability of accepting as correct the conclusion that group II had more DNA replication forks containing ferritin-labeled antibodies at the junction region than group I.

Table 1. Electron microscopic analysis of apparent replication fork structures

	Group I	Group II
Incubation following initial B[*a*]P diol epoxide-I exposure, min	70*	80
Apparent DNA replication fork structures, total no.	147	161
Fork structures tagged with ferritin-labeled antibodies, no.	30 (20%)	43 (27%)
Fork structures with ferritin-labeled antibodies at junction region, no.	12 (8%)	21 (13%)

DNA was purified from C3H/10T½ Cl8 cells exposed to 3.3 μM B[*a*]P diol epoxide-I in mid-S phase and incubated with primary and secondary antibodies. The χ^2 for the difference between group I and group II in the number of DNA replication forks with ferritin-labeled antibodies at the junction region was 1.913. For one degree of freedom, $\chi^2_{0.90} = 2.0706$ and $\chi^2_{0.80} = 1.642$. Values in parentheses represent percent of total.
*Replication inhibited.

The distribution of ferritin-labeled antibodies bound to carcinogen-DNA adducts on DNA replication forks was analyzed for group I and group II. First, the replicated branches of the DNA replication forks were identified by their near-equal length from measurements of electron micrographs. Second, a junction region was designated as composed of three joint segments of 80 bp each that delimited an area of 160 bp in diameter, roughly corresponding to the diameter of the ferritin molecule. Third, we elected to divide the length of each branch, away from the junction region, into 230-bp segments. This distance corresponds approximately to the expected internucleosomal spacing (26). Table 2 lists the DNA segments (distances from the fork junction region in base pairs) analyzed for group I and group II fork structures, the number of DNA molecules analyzed that contained each of these segments, and the total number

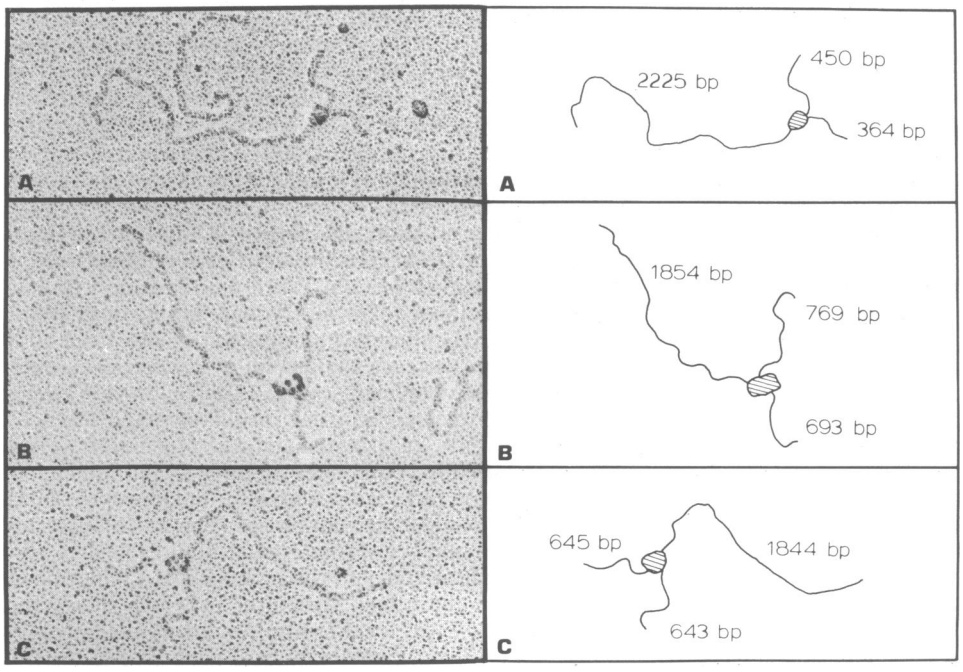

FIG. 2. Electron microscopic analysis of DNA modified by B[*a*]P diol epoxide-I. C3H/10T½ cells were treated with B[*a*]P diol epoxide-I for 10 min in mid-S phase and incubated for 70 min in fresh medium (group II). DNA was purified, digested with restriction enzyme, incubated with primary rabbit (anti-B[*a*]P diol epoxide-I-DNA) antiserum, digested with papain, and incubated with ferritin-labeled monovalent secondary antibody fragments. Replication forks containing attached antibodies were detected by electron microscopy and photographed. Tracings of the replication fork structures in the micrographs to the left (*A–C*) are on the right with the estimated length of each DNA branch.

of ferritin-labeled antibodies located in each segment. By dividing the total number of observed ferritin-labeled antibodies by the total number of DNA segments listed in Table 2, a frequency of ferritin-labeled antibodies per 230-bp segment of DNA was determined for each treatment group. By multiplying the number of molecules containing each DNA segment by this calculated frequency, an expected number of ferritin-labeled antibodies per DNA segment was determined, assuming a random, equal distribution of ferritin-labeled antibodies throughout the DNA molecules. The observed frequency distribution had zero values in a number of locations on both parental and daughter branches. To perform a χ^2 test, data were grouped into four larger segments along the lengths of the DNA fragments to eliminate these zero values. The χ^2 analysis showed that the distribution of ferritin-labeled antibodies was not a random distribution on the DNA replication forks with $P < 0.0005$. To be sure that the χ^2 test was not improperly biased by the grouping, a logistic regression analysis was also performed on the data in Table 2. This procedure is little affected by the small number of counts in the frequency distribution. The model assumed that the ferritin-labeled antibodies were distributed binomially along the length of the DNA. In concordance with the χ^2 test, the results of the logistic regression confirmed that a higher proportion of ferritin-labeled antibodies were found at the junction region of the fork at a highly significant level of $P < 0.0001$. As shown in Table 1, of the total number of ferritin-labeled antibodies bound to DNA replication fork structures, 12 of 30 (40%) in group I and 21 of 43 (49%) in group II were localized to the junction region of the fork structures.

DISCUSSION

The results presented in this paper demonstrate that DNA in replication forks does not show a random pattern of adduction by the carcinogen B[*a*]P diol epoxide-I. The observed distribution of B[*a*]P diol epoxide-I adducts seems to reflect the vulnerability of replicating DNA to carcinogen binding and the effects of carcinogen adducts in DNA on the further progress of DNA replication. Our analysis was limited to DNA structures identifiable by electron microscopy as replication forks according to the criteria listed in *Materials and Methods*. We have not attempted to quantify total number of forks per unit length of DNA nor to identify possible carcinogen-induced abnormalities in fork structures.

The two experimental groups reported here differ based upon the extent of progression of DNA replication following carcinogen treatment. Cell cultures were allowed to replicate DNA for 70 min after carcinogen treatment (group II). As a control for the effect of DNA replication, parallel cultures were incubated with aphidicolin before, during, and after exposure to B[*a*]P diol epoxide-I to slow the displacement of DNA replication forks (group I).

We have estimated the probability of an active replication fork encountering a B[*a*]P diol epoxide-I-DNA adduct for each treatment group based on rates of DNA synthesis and levels of B[*a*]P diol epoxide-I modification of DNA. The level of modification for DNA from group II was 40.4 fmol of B[*a*]P diol epoxide-I-DNA adduct per μg of DNA (26.9 adducts in 10^6 bp). Therefore, the average interadduct distance, assuming a random distribution of adducts, would be 37 kilobases (kb) of DNA. Thus, the average distance traversed by an initiated fork, from the time of treatment until it encounters an adduct, would be between 18 kb and 56 kb (0.5–1.5 times the interadduct distance). Cordeiro-Stone and Kaufman (27) found the rate of DNA strand growth in 10T½ cells to be 1.2×10^6 Da/min or ≈1.8 kb/min per fork. Thus, the average time of replication per growing point, prior to reaching a blocking lesion in the template strand, would be 10–31 min. If this estimate is correct, one would expect the majority of the replication forks to be blocked by an adduct. However, for those origins that were resistant to the inhibition of replicon initiation by B[*a*]P diol epoxide-I (20) and were activated during the 70-min incubation period, as much as 50% of the initiated forks may not have reached a blocking adduct. Also, a fraction of the replication forks may never encounter an adduct, because some replicons may be smaller than the interadduct distance.

Cell cultures of group I were incubated with aphidicolin at 2.0 μg/ml before, during, and after exposure to B[*a*]P diol epoxide-I to inhibit DNA synthesis. Under these conditions, the rate of DNA strand growth was found to be 1.2×10^5

Table 2. Distribution of ferritin-labeled antibodies bound to sites of carcinogen modification on DNA replication fork structures

DNA segment(s), bp from junction	Group I			Group II		
	DNA molecules, total no.	Ferritin-labeled antibodies, total no.		DNA molecules, total no.	Ferritin-labeled antibodies, total no.	
		Obs.	Exp.		Obs.	Exp.
Parental branch						
1231–1460	22	0	1.2	32	2	1.8
1001–1230	23	0	1.2	33	0	1.9
771–1000	26	0	1.4	35	0	2.0
541–770	28	0	1.5	37	0	2.1
311–540	30	1	1.6	40	0	2.3
81–310	30	1	1.6	41	1	2.4
Fork junction						
0–80 in all 3 branches	30	12	1.6	43	21	2.5
Daughter branches						
81–310	60	4	3.2	86	2	5.0
311–540	60	1	3.2	82	4	4.7
541–770	52	1	2.8	70	3	4.0
771–1000	44	2	2.4	52	0	3.0
1001–1230	42	3	2.3	38	2	2.2
1231–1460	38	1	2.0	36	1	2.1
Total	485	26	26	625	36	36

Analysis done on fork structures tagged with ferritin-labeled antibodies. Obs., observed; Exp., expected.

Da/min or 0.36 kb/min (27). The DNA was modified by B[*a*]P diol epoxide-I to 39.3 fmol of adduct per μg of DNA (25.9 adducts in 10^6 bp). With an average interadduct distance of 39 kb, the estimated average distance traversed by a replication fork before being blocked by an adduct again would be 18–56 kb; however, in the presence of aphidicolin, at a reduced rate of fork displacement (0.18 kb/min per fork), it would take 10 times longer for the majority of the forks to be blocked. Since each fork could only traverse ≈11 kb in 60 min in the presence of aphidicolin, of those forks initiated before carcinogen treatment, 20% (11/56) to 61% (11/18) could still be blocked at a B[*a*]P diol epoxide-I adduct. Again, these figures are upper limits, because we have not considered those replication forks that were initiated during the 60-min incubation after carcinogen treatment. We have observed that aphidicolin will not inhibit the initiation of replicons, although it is a powerful inhibitor of DNA strand growth (27). On the other hand, B[*a*]P diol epoxide-I treatment is an effective inhibitor of replicon initiation (19, 20), so this effect of newly initiated replication forks is probably small.

Biochemical studies with mammalian cells in culture have demonstrated a dose-dependent decrease in the rate of DNA synthesis by B[*a*]P diol epoxide-I (13, 18–21), in a manner similar to that observed in cells treated with ultraviolet light (28, 29). The smaller nascent DNA synthesized in cells treated with ultraviolet light or B[*a*]P diol epoxide-I are thought to be the consequence of the blockage of replication forks at lesions present in the template to the leading strand, and the formation of daughter-strand gaps opposite lesions in the template for the lagging strand (29). In the present study we observed a statistically significant number of replication forks containing ferritin-labeled antibody complexes associated with the junction region. This localization is consistent with the blockage of replication forks by B[*a*]P diol epoxide-I adducts present in the template to the leading strand. Furthermore, we observed four to six times more adducts in the two daughter branches than in the parental branch (excluding those in the fork junction). These adducts found in replicated DNA were probably located in DNA strands that serve as template for the synthesis of the lagging strand. They would be expected to block the elongation of Okazaki fragments but not to interrupt the displacement of the replication fork (29).

The results of electron microscopic visualization of antibodies bound to B[*a*]P diol epoxide-I-DNA adducts (Table 1) revealed that 27% of the observed DNA replication forks had antibodies attached to them in group II, as compared to the 20% observed when aphidicolin was present (group I). Within this population of DNA replication forks labeled with antibodies, a statistically significant portion of the antibodies were found at the junction region (Table 2). From the total number of DNA replication forks with ferritin-labeled antibodies associated with them, 40% in group I (12/30) and 49% in group II (21/43) had antibodies visualized at the fork junction region.

These results indicate that the increased localization of adducts at the fork junction cannot be explained only by the accumulation of forks arrested at an adduct when replication has reached this location. Whether replication progressed fast or slowly, most adducts were found in the junctional region of the fork. This suggests that a large fraction of the carcinogen binding occurred directly to DNA at the fork junction, presumably because of the structure of the fork and the specific vulnerability of replicating DNA (30, 31). The persistence of this fraction of binding independent of the extent of replication suggests that many of these sites of adduction blocked further replication. The fact that less than a majority of forks have a demonstrable adduct may relate to (*i*) less than complete identification of adducts by antibody, (*ii*) adduction at the fork junction may increase the probability of fork breakage, (*iii*) repair processes may remove adducts ahead of replication forks, (*iv*) the replicon size in C3H/10T½ cells may be smaller than 60–135 kb, the range of replicon sizes reported for rodent cells (32). Despite these possible confounding factors, our results indicate that the junction region of replication forks is particularly vulnerable to adduction by B[*a*]P diol epoxide-I and that DNA adducts block the displacement of replication forks. This may explain, in large measure, the vulnerability of proliferating cells to transformation.

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