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Genetics

Effect of excision repair by diploid human fibroblasts on the kinds and locations of mutations induced by (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene in the coding region of the *HPRT* gene

(nucleotide excision repair/cell cycle effects/mutational spectra/thioguanine resistance/human cells)

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ABSTRACT **(±)-7β,8α-Dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE) is a direct-acting carcinogen that forms DNA adducts only with purines, predominantly (>95%) with guanine. To investigate the effect of nucleotide excision repair on the kinds and locations (spectra) of mutations induced in diploid human fibroblasts by BPDE, we synchronized cells and exposed them to BPDE either at the beginning of S phase just when the target gene hypoxanthine (guanine) phosphoribosyltransferase (*HPRT*) is replicated or 12 hr prior to the beginning of S phase (early G_1 phase). Clones resistant to 6-thioguanine were isolated, and the mRNA in lysates of 100–500 cells from each mutant clone was used to synthesize cDNA. *HPRT* cDNA was amplified 10^{11}-fold by the polymerase chain reaction and then sequenced directly. The mutants derived from the two populations did not differ in the *kinds* of mutations; 19/20 of the base substitutions in cells taken from S phase and 19/19 of those from G_1 phase involved G·C base pairs, predominantly G·C → T·A. However, they differed significantly in the distribution of the mutations in the coding region of the gene. In the cells from G_1 phase, 29% of the mutations were clustered within a unique run of six guanine bases; in the S-phase cells, only 4% were located there. Assuming that the premutagenic BPDE-induced lesions involved purines, in the cells treated at the beginning of S phase, 24% of these lesions were located in the transcribed strand, whereas in the G_1-treated cells, none were. This suggests that in the *HPRT* gene of diploid human cells excision repair of BPDE adducts occurs preferentially on the transcribed strand.**

In previous studies, we and our colleagues investigated the relationship of DNA repair and DNA replication to mutagenesis induced by carcinogens in diploid human fibroblasts. We found that the frequency of mutants induced by UV radiation (1, 2), (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE) (3, 4), or 1-nitrosopyrene (5) in nucleotide excision repair-proficient cells is highest in cells treated at early S phase and much lower in cells treated in early G_1 phase. In cells from xeroderma pigmentosum (XP) patients, which are virtually incapable of nucleotide excision repair, the frequency of induced mutants in cells treated in G_1 phase is the same as in cells treated at S (2, 3). These data suggest that repair processes play a key role in the cell cycle-related sensitivity to mutagenicity observed in synchronized repair-proficient cells and that DNA replication is centrally involved in the conversion of DNA damage into mutations.

The effect of the cell cycle on the frequency of transformation has also been determined in diploid human cells (6) and rodent cell lines (7). Populations treated in early S phase exhibit a higher frequency of transformed cells than populations exposed in early G_1 phase (6, 7). No difference was seen with XP cells (6). This higher frequency of transformants in populations treated in early S phase might simply reflect the higher frequency of mutations induced. However, it might result from a difference in the *spectrum* (i.e., kinds and locations) of mutations induced in the two populations, so that a specific mutation required for transformation (e.g., leading to the activation of a cellular protooncogene) occurs preferentially when cells are treated in S phase.

To our knowledge there are no published studies that have determined whether cells treated in S phase and cells treated in G_1 differ in the *type* and/or *location* of mutations induced. However, Hanawalt and Bohr and their colleagues showed that in certain mammalian cell lines the rate of nucleotide excision repair of pyrimidine dimers in transcriptionally active genes differs from that occurring in nonactively transcribed genes (8–11). Mellon *et al.* (12) showed that in a Chinese hamster cell line (CHO) and in a human cell line (6A3) the rate of excision repair of damage in the transcribed strand of the gene for dihydrofolate reductase (*dhfr*) is more rapid than in the nontranscribed strand. If such differential repair occurs in the hypoxanthine (guanine) phosphoribosyltransferase (*HPRT*) gene of diploid human cells and if mutations are introduced by the DNA replication occurring in S phase, significant differences should be seen between the mutational spectra of synchronized cells exposed to DNA-damaging agents just as S-phase DNA replication of a target gene is taking place and those exposed in early G_1. To test this hypothesis, we treated repair-proficient human fibroblasts with BPDE in early S phase or in early G_1 and determined the kinds and locations of mutations induced in the coding region of the *HPRT* gene. The results are consistent with the hypothesis: the two populations showed no difference in the kinds of mutations induced by BPDE, but the location of the mutations differed significantly.

MATERIALS AND METHODS

Cells and Media. Diploid human cells derived from neonatal foreskin material (13) were cultured in modified McM medium (14) prepared with Earle's salts and containing 10% supplemented calf serum (HyClone) (culture medium). For selection of 6-thioguanine-resistant cells, the same medium, but lacking adenine and containing 5% (vol/vol) supple-

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Abbreviations: BPDE, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; XP, xeroderma pigmentosum; G_0 phase, resting, noncycling stage; G_1 phase, period in the cell cycle between mitosis and S phase; S phase, semiconservative DNA synthesis period in the cell cycle.

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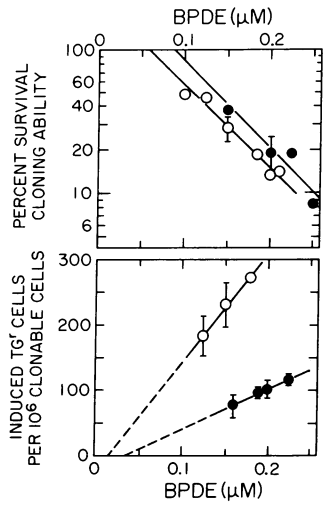

FIG. 1. Cytotoxicity (*Upper*) and frequency of mutations to 6-thioguanine resistance (TG^r) (*Lower*) induced by BPDE in human cells treated in early S phase (○) or 12 hr prior to the onset of S phase (●). The frequencies have been corrected for the cloning efficiency (40%–60%) of the cells at the time of selection. The background frequencies, which range from 5×10^{-6} to 15×10^{-6} have been subtracted. The lines shown were determined by the least-squares method.

mented calf serum (HyClone), 5% fetal calf serum, and 40 μM 6-thioguanine, was used.

Cell Synchronization. Cells were inoculated at a density 8- to 10-fold less than that attained at confluence and refed culture medium every other day to stimulate rapid growth to confluence. They were then fed daily for 3 additional days to allow them to fill the dish to capacity. The medium was not changed for the next 72–96 hr so that the cells would cease proliferation and enter the G_0 state. Autoradiography studies showed that in such cultures DNA synthesis was virtually eliminated (2). To stimulate the cells to enter the cell cycle, they were plated in culture medium at a density of 10^4 cells per cm^2. Previous studies have shown that such cells begin DNA synthesis (S phase) after 16–17 hr (4) and that at least 80% of the cells are at S phase after 4 additional hr (2).

Exposure to Mutagen and Isolation of *HPRT* Mutants. The details of the mutagenesis assay have been described (4, 5). Briefly, a series of independent populations (1.5×10^6 cells each, plated in 150-mm diameter dishes) were treated 17 hr after release from G_0 (at the beginning of S phase) or 4 hr after release from G_0 (in early G_1 phase). The culture medium was replaced with serum-free medium. BPDE, freshly dissolved in anhydrous dimethyl sulfoxide, was delivered into the serum-free medium by a micropipette. After 1 hr, the medium was removed, and the cells were rinsed with phosphate-buffered saline (pH 7.4) and refed with fresh culture medium. The cells in one dish were assayed immediately for survival of colony-forming ability as described (15). The rest were allowed 8 days for expression of resistance to 6-thioguanine before 0.5×10^6 cells from each population were plated in selective medium. When macroscopic drug-resistant clones developed 14 days later, they were located, isolated, and expanded.

Synthesis of First-Strand cDNA Directly from mRNA in Cells. Cells were trypsinized and suspended in culture medium, the cell number was determined by electronic counting, and the cells were diluted in cold phosphate-buffered saline (pH 7.4). From 100 to 500 cells were transferred to a

Table 1. Kinds and locations of the mutations induced in the coding region of the *HPRT* gene in cells treated with BPDE in S phase of the cell cycle

Mutant	Position	Exon	Type of mutation	Surrounding sequence (coding strand)*	Amino acid change	Strand with the affected purine
			Base substitution			
BPS16†	88	2	G·C → T·A	GCT GAG GAT	Glu → Stop	NT
BPS87	97	2	G·C → T·A	TTG GAA AGG	Glu → Stop	NT
BPS28	119	2	G·C → T·A	CAT GGA CTA	Gly → Val	NT
BPS51	119	2	G·C → T·A	CAT GGA CTA	Gly → Val	NT
BPS4	130	2	G·C → T·A	ATG GAC AGG	Asp → Tyr	NT
BPS66	134	2	G·C → T·A	GAC AGG ACT	Arg → Met	NT
BPS3	149	3	G·C → T·A	CTT GCT CGA	Ala → Asp	T
BPS16†	178	3	G·C → T·A	GGC CAT CAC	His → Asn	T
BPS21	178	3	G·C → T·A	GGC CAT CAC	His → Asn	T
BPS23	178	3	G·C → T·A	GGC CAT CAC	His → Asn	T
BPS94	212	3	G·C → T·A	AAG GGG GGC	Gly → Val	NT
BPS47	229	3	G·C → T·A	GCT GAC CTG	Asp → Tyr	NT
BPS34	337	4	G·C → T·A	GGG GAC ATA	Asp → Tyr	NT
BPS78	438	6	G·C → C·G	ACT TTG CTT	Leu → Phe	NT
BPS83	447	6	G·C → C·G	TCC TTG GTC	Leu → Phe	NT
BPS75	451	6	A·T → G·C	GTC AGG CAG	Arg → Gly	NT
BPS13	471	6	G·C → T·A	AAG ATG GTC	Met → Ile	NT
BPS62	478	6	G·C → A·T	AAG GTC GCA	Val → Ile	NT
BPS95	531	7	G·C → T·A	CCA GAC TTT	Asp → Glu	T
BPS41	568	8	G·C → T·A	GTA GGA TAT	Gly → Stop	NT
			Putative splice site mutation			
BPS72	28–134	2	Exon 2 missing			NA
BPS32	319–384	4	Exon 4 missing			NA
BPS69	486–532	7	Exon 7 missing			NA
			Other			
BPS37	477 or 478	6	Deletion of a G	AAG GTC GCA	Frameshift	NT

T, transcribed; NT, nontranscribed; NA, not applicable.
*The base that was altered is underlined.
†Mutant with two base substitutions.

0.5-ml Eppendorf tube and centrifuged for 10 min at 4°C. The supernatant was removed, and the cell pellet was resuspended in 5 μl of the cDNA cocktail described by Yang *et al.* (16). The reverse transcriptase reaction was performed at 37°C for 1 hr to allow the cell membranes to be lysed by detergent and first-strand cDNA to be synthesized from total poly(A)-mRNA.

Amplification of *HPRT* cDNA and DNA Sequencing. The experimental conditions, optimized for preparing second-strand *HPRT* cDNA, amplifying the cDNA 10^{11}-fold by using two polymerase chain reactions (30 cycles each), and sequencing the product directly by a modified Sanger dideoxynucleotide procedure using three sequencing primers have been described (16).

RESULTS

Cytotoxicity and Frequency of Mutants Induced by BPDE in Cells Treated in the G_1 Phase or S Phase of the Cell Cycle. Individual populations of cells were synchronized and exposed to various concentrations of BPDE in early S phase or in early G_1. The cells were assayed for survival and for the frequency of 6-thioguanine-resistant cells (Fig. 1). As expected (3), there was little difference in the sensitivity of the two populations to the cytotoxic effects of BPDE, indicating that the initial level of DNA adduct formation in the two populations was comparable (17). In contrast, the frequency of mutants induced in populations treated in early G_1 phase was 3-fold lower than that of cells treated in early S. These results support the hypothesis that S-phase DNA replication is responsible for converting BPDE-induced lesions into mutations since cells treated in G_1-phase will be able to excise a higher percentage of BPDE-induced adducts than will cells treated in S phase. It should be pointed out that, in each experiment, the frequency of 6-thioguanine-resistant cells in the populations treated in early S phase ranged from 30 to 50 times background; in the populations treated in the G_1 phase, it ranged from 4 to 16 times background. To decrease the chances of including a mutant containing a spontaneous mutation, rather than a BPDE-induced mutation, we only used data from experiments in which the frequency was at least 10 times higher than background.

Characterization of *HPRT* Mutants. To determine the nature of the BPDE-induced mutations at the DNA sequence level, 23 drug-resistant clones were isolated from cells treated in S phase, and 23 drug-resistant clones were isolated from cells treated in G_1 phase. The cells were tested for inability to grow in medium containing hypoxanthine (0.1 mM), aminopterin (2 μM), and thymidine (30 μM), in order to confirm that they lacked a functional *HPRT* gene. Then cDNA was synthesized from the total mRNA of a lysate of 100–500 cells from each clone, and the cDNA of the *HPRT* gene was amplified 10^{11}-fold and sequenced directly. The results are shown in Tables 1 and 2. All 23 mutants listed from each phase represent unequivocally independent mutants, either because their alterations were unique or because they were derived from independent populations of treated cells.

There was no significant difference between S- and G_1-phase-derived mutants in the kinds of mutations they contained (Tables 1 and 2). Nineteen out of 23 S-phase mutants and 18 out of the 23 G_1-phase mutants contained base substitutions; 1 mutant from each phase exhibited a second base substitution at a site distant from the first. Of the remaining mutants, 3 from each phase may also have contained base substitutions in a 5′ or 3′ consensus splice site, since one of the nine exons was completely deleted from their cDNA, but no

Table 2. Kinds and locations of the mutations induced in the coding region of the *HPRT* gene in cells treated with BPDE in G_1 phase of the cell cycle

Mutant	Position	Exon	Type of mutation	Surrounding sequence (coding strand)*	Amino acid change	Strand with the affected purine
			Base substitution			
BPG22	88	2	G·C → T·A	GCT GAG GAT	Glu → Stop	NT
BPG7	97	2	G·C → T·A	TTG GAA AGG	Glu → Stop	NT
BPG113	130	2	G·C → T·A	ATG GAC AGG	Asp → Tyr	NT
BPG29	134	2	G·C → T·A	GAC AGG ACT	Arg → Met	NT
BPG109	139	3	G·C → A·T	ACT GAA CGT	Glu → Lys	NT
BPG107	208	3	G·C → A·T	AAG GGG GGC	Gly → Arg	NT
BPG101	211	3	G·C → C·G	AAG GGG GGC	Gly → Arg	NT
BPG19	211	3	G·C → T·A	AAG GGG GGC	Gly → Cys	NT
BPG12	212	3	G·C → T·A	AAG GGG GGC	Gly → Val	NT
BPG13	212	3	G·C → T·A	AAG GGG GGC	Gly → Val	NT
BPG44	212	3	G·C → A·T	AAG GGG GGC	Gly → Asp	NT
BPG64	212	3	G·C → T·A	AAG GGG GGC	Gly → Val	NT
BPG77	229	3	G·C → T·A	GCT GAC CTG	Asp → Tyr	NT
BPG48	229	3	G·C → T·A	GCT GAC CTG	Asp → Tyr	NT
BPG8†	388	5	G·C → C·G	AAT GTC TTG	Val → Leu	NT
BPG76	393	5	G·C → C·G	GTC TTG ATT	Leu → Phe	NT
BPG18	419	6	G·C → T·A	ACT GGC AAA	Gly → Val	NT
BPG8†	529	7	G·C → T·A	CCA GAC TTT	Asp → Tyr	NT
BPG112	535	8	G·C → T·A	TTT GTT GGA	Val → Phe	NT
			Putative splice site mutation			
BPG10	28–134	2	Exon 2 missing			NA
BPG116	319–384	4	Exon 4 missing			NA
BPG9	403–485	6	Exon 6 missing			NA
			Other			
BPG17	98–100	2	Insertion of an A	TGG GAA AGG	Frameshift	NT
BPG100	535	8	Deletion of a G	TTT GTT GGA	Frameshift	NT

T, transcribed; NT, nontranscribed; NA, not applicable.
*The base that was altered is underlined.
†Mutant with two base substitutions.

Table 3. Types of base substitutions observed

Type of base substitution	No. of substitutions observed	
	G_1 phase	S phase
	Transversion	
G·C → T·A	13	16
G·C → C·G	3	2
	Transition	
G·C → A·T	3	1
A·T → G·C	0	1
Total	19	20

other change was seen. In addition 1 mutant from each phase contained a G·C base pair deletion, and one G_1-phase mutant exhibited an A·T base pair insertion. Table 3 shows that there also was no significant difference in the kinds of base substitutions observed in the mutants obtained from the two populations. All except one S-phase-derived mutant involved G·C base pairs. Transversion of G·C → T·A occurred predominantly: 80% for S phase and 68% for G_1 phase. Fig. 2 diagrams the locations of the mutations. Note that a substantial fraction of the mutations were located in exon 2 or the first half of exon 3.

Strand Distribution of the Premutagenic Lesions Induced by BPDE. The fact that in cultured mammalian cells BPDE binds only to purines, with >95% of the DNA adducts involving guanine (18, 19), allows one to infer which strand contained the premutagenic lesions that resulted in the observed mutations. As noted in Tables 1 and 2 and diagrammed in Fig. 2, the mutants obtained from populations treated in S phase exhibited mutations for which the premutagenic lesions were located in either strand (i.e., 24% were in the transcribed strand). In contrast, in the mutants from populations treated in early G_1 and allowed at least 12 hr for excision repair before DNA synthesis began, 100% of the premutagenic lesions were located in the nontranscribed strand. This difference in strand distribution (24:76 versus 0:100) is statistically significant, with $P < 0.05$ using the χ^2 test.

Mutational "Hot Spot Region" Obtained from *HPRT* Mutants in Cells Exposed in G_1 Phase. One very strong mutational hot spot region was found in the spectrum of mutations induced in cells treated in early G_1 phase (Fig. 2). Seven out of 24 mutations (29%) occurred within positions 208–212 (Table 2) in a unique region containing six consecutive guanine bases flanked by adenine on the 5′ side and cytosine on the 3′ side. In contrast, only 1 out of 24 mutations (4%) induced in cells treated at S phase occurred in this region (Table 1).

DISCUSSION

Although the mutant frequency in cells treated in G_1 phase was 3 times lower than that in cells treated in early S phase, the *kinds* of mutations induced were similar. Our finding that BPDE caused base substitutions almost exclusively at G·C base pairs suggests that mutagenesis is targeted to adducts. These data agree with the results observed in the *lacI* gene of *Escherichia coli* (20), in an extrachromosomal *supF* gene (21), and in several endogenous genes of rodent cell lines (22, 23).

The predominance of G·C → T·A substitutions may be the result of the DNA polymerases preferentially inserting an adenine base opposite a noninstructional base containing a bulky BPDE adduct (24). Another possible explanation for this predominance is that the presence of a BPDE adduct on guanine allows the formation of stable purine·purine base pairing, with one of the purines in the *syn* position (25, 26), and that this mispairing is not recognized.

The expected level of DNA adduct formation by BPDE at a concentration yielding ≈10% survival is ≈40 per 10^6 base pairs (3) (i.e., 2 per 44,000 base pair *HPRT* gene). Therefore, it is not surprising to find that 2 mutants out of 46 analyzed (BPS16 and BPG8) contained two independent mutations.

Three of the 23 mutants derived from either phase of the cell cycle produced *HPRT* mRNA in which one of the exons was completely lacking. These mutants may contain a point mutation in the 5′ or 3′ consensus splice site so that the splicing machinery cannot recognize the site and splicing occurs using the nearest neighbor site.

Mellon *et al.* (12) recently reported a significant difference in the efficiency of removal of UV-induced pyrimidine dimers from the two strands of the *dhfr* gene in cultured hamster and human cells; the transcribed strand was preferentially repaired in both species. Vrieling *et al.* (27) investigated UV-induced *hprt* mutants in a hamster cell line (V79) and proposed preferential repair of the transcribed strand as one explanation for the strand specificity of the mutations in this cell line. If one assumes that the premutagenic lesions induced by BPDE involve purine adducts, then our data in Tables 1 and 2 and Fig. 2 support the hypothesis that, in diploid human fibroblasts, nucleotide excision repair of BPDE-induced adducts occurs preferentially on the transcribed strand. None of the 21 non-splice-site mutations observed in the mutants taken from G_1 phase, where the cells had at least 12 hr for excision repair before DNA replication began, had a purine in the transcribed strand. In contrast, 24% of those derived from S phase did. The data of Mazur and Glickman (22) in the *aprt* gene of asynchronously growing, excision repair-proficient Chinese hamster ovary cells, that of Carothers and Grunberger (23) in the *dhfr* gene of such cells, and that of Yang *et al.* (28) in the *HPRT* gene of asynchronously growing human cells also support this hypothesis. However, unlike the present study, those investigators did not compare or contrast two populations of cells.

The distribution of guanine bases in the coding region of the human *HPRT* gene is 41% in the transcribed strand and 59% in the nontranscribed strand, but this ratio becomes 38:62 when those guanines that will not cause an amino acid change, even if mutated, are taken into consideration. If one assumes that binding of BPDE to guanine bases in the *HPRT*

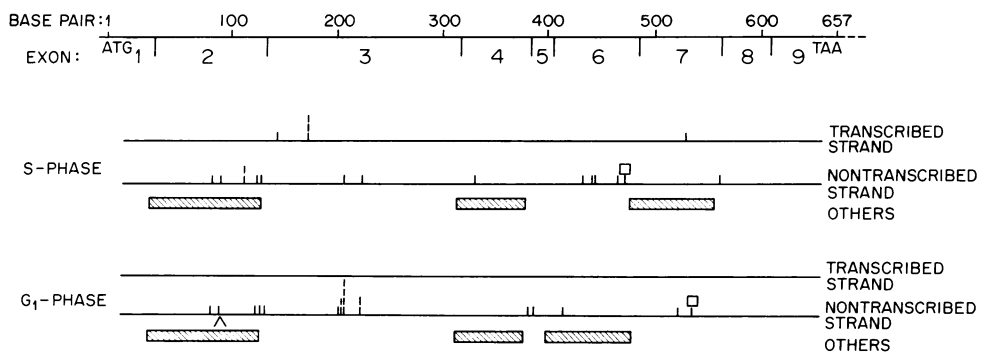

FIG. 2. The location of mutations and the strand distribution of the guanine bases involved in a base change induced by BPDE in the coding region of *HPRT* gene. The positions of the ATG start codon, TAA stop codon, and the nine exons are indicated. Vertical lines, base substitutions; square, a deleted base pair; caret, an inserted base pair; rectangles, deleted exons.

gene is random and that virtually every amino acid change renders a cell resistant to thioguanine, in cells that have no time to excise BPDE-induced damage, 38% of the mutations should correspond to a guanine in the transcribed strand. Only 24% of the mutations seen in the cells treated in early S phase had this characteristic. There is no evidence that BPDE binds more to the purines in one strand of a particular gene than in the other. Bias caused by silent mutations is a far more likely explanation and the one that we favor. For example, a high proportion of the mutations analyzed were located in exon 2 and the first half of exon 3, the part of the gene that codes for the active sites in this enzyme (29). Of course, since treatment of cells with BPDE temporarily stops DNA replication (30), it could be that by the time replication resumes some fraction of the adducts have been removed by nucleotide excision repair. This would change the ratio. Evidence against this explanation for our finding that only 25% of the mutations in the S phase cells involved a guanine in the transcribed strand comes from our finding that the percentage of such mutations in excision repair-deficient XP cells (complementation group A) treated in early S was 28% and in early G_1 phase was 24% (unpublished studies).

Mazur and Glickman (22) showed that a 5′-AG(G)$_n$A-3′ sequence was a hot spot region for mutations induced by BPDE in the *aprt* gene of Chinese hamster ovary cells. In the coding region of the human *HPRT* gene, there are nine such sequences that are not interrupted by splicing junctions. Analysis of our sequence data indicates that only 1 out of 24 mutations from G_1-phase cells and 3 out of 24 from S-phase cells occurred in such sequences. In contrast, our study revealed a cell-cycle dependent hot spot region in the sequence 5′-A(G)$_6$C-3′ (positions 206–213). Seven out of the 24 mutations induced in G_1 phase (29%) were located in this region, compared with 1 out of 24 from S-phase cells (4%) (Fig. 2 and Tables 1 and 2). Although it has been reported that certain poly(dG) sequences are the preferred sites for BPDE binding (31, 32), this cannot explain the cell-cycle dependent feature of the very prominent hot spot region that we observed. It is more likely that this hot spot region seen in the G_1 cells resulted from inefficient repair of BPDE adducts in the run of six consecutive guanine bases so that after excision repair had occurred for a certain period of time, the adducts remaining in this region represented a higher fraction than originally present. Support for this hypothesis comes from studies showing that the helix of poly(dG)·poly(dC) prefers an A-like DNA helix geometry, rather than the traditional B form (33, 34). If this run of guanine bases assumes a specific local DNA conformation, then this could perhaps diminish the efficiency of excision repair of the BPDE adducts in this region. If inefficient repair accounts for the hot spot region seen only in the G_1 cells, it should not be a prominent hot spot region in the spectrum of the XP cells from complementation group A.

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