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Reversion Analysis of Mutations Induced by 5-Bromodeoxyuridine Mutagenesis in Mammalian Cells

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Two protocols have been developed, both of which utilize the thymidine analog 5-bromodeoxyuridine (BrdUrd) to induce mutations in mammalian cells in culture (E. R. Kaufman and R. L. Davidson, Proc. Natl. Acad. Sci. USA 75:4982–4986, 1978; E. R. Kaufman, Mol. Cell. Biol. 4:2449–2454, 1984). The first protocol, termed incorporational (INC) mutagenesis, utilizes high concentrations of BrdUrd in the culture medium to generate a high intracellular ratio of BrdUTP/dCTP. The second protocol, termed replicational (REP) mutagenesis, entails the incorporation of BrdUrd into DNA under nonmutagenic conditions, the removal of all BrdUrd from the culture medium, and the subsequent replication of the bromouracil-containing DNA in the presence of high intracellular levels of dTTP and dGTP. Genetic studies using reversion analysis at the hypoxanthine-guanine phosphoribosyltransferase locus were used to determine whether the mechanisms of these two BrdUrd mutagenesis protocols had enough specificity to be distinguishable by their ability to revert various mutants. The results of these studies indicated that (i) mutants induced by INC mutagenesis were induced to revert only by REP mutagenesis and not by INC mutagenesis, (ii) mutants induced by REP mutagenesis were more efficiently reverted by INC mutagenesis than by REP mutagenesis, and (iii) both spontaneous mutants and mutants induced by the chemical mutagen ethyl methanesulfonate showed a high degree of specificity when tested for reversion by the BrdUrd mutagenesis protocols.

The thymidine (dThd) analog 5-bromodeoxyuridine (BrdUrd) has been shown to be an effective mutagen in procaryotic (3, 10, 16, 20, 23) and eucaryotic (1, 11, 13, 22) systems. A model (10) for the mutagenic action of this base analog suggested that due to its chemical properties, 5-bromouracil (BrUra) would undergo a tautomeric shift to the rare enol state, allowing it to mispair with guanine more frequently than would thymine. Thus, two possible mechanisms for BrUra mutagenesis were proposed, errors of incorporation and errors of replication. Errors of incorporation were thought to occur when BrdUTP mispaired with a guanine residue in replicating DNA, resulting in a G · C-to-A · T transition after further replication. Errors of replication were thought to occur when a BrUra residue in replicating DNA mispaired with dGTP, resulting in A · T-to-G · C transitions.

Studies in mammalian systems initially suggested that BrdUrd mutagenesis was determined by the concentration of BrdUrd to which the cells were exposed and was independent of the amount of BrUra incorporated into DNA in place of thymine residues (13). These and other findings (2, 7, 8, 14) provided evidence that (i) BrdUrd mutagenesis in mammalian cells was driven by high concentrations of BrdUrd which caused an increase in the intracellular ratio of BrdUTP/dCTP, (ii) BrdUrd mutagenesis occurred only during incorporation of BrdUTP into DNA, and (iii) mutations induced by the replication of BrUra residues in DNA did not occur. (This type of BrdUrd mutagenesis will be termed incorporational or INC mutagenesis.)

More recent studies with mammalian cells (12) have established a new BrdUrd mutagenesis protocol which has allowed the unambiguous demonstration of mutations induced by the replication of BrUra residues in DNA. This new protocol involves the incorporation of BrUra into DNA under nonmutagenic conditions, the removal of the BrdUrd from the culture medium, and the subsequent replication of the BrUra-containing DNA in the presence of high intracellular levels of dTTP and dGTP. (This type of BrdUrd mutagenesis will be termed replicational or REP mutagenesis.)

The experiments presented here sought to test, by reversion analysis, whether the two BrdUrd mutagenesis protocols actually act via different mechanisms. The rationale of these experiments was that mutations induced by INC mutagenesis would show a pattern of reversion different from that of mutations induced by REP mutagenesis. A number of mutants resistant to 6-thioguanine (Sgu) and, therefore, probably mutated at the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) locus were induced by each of the mutagenesis protocols. Then, each of the two BrdUrd mutagenesis protocols was tested for its ability to revert each of the mutants. The results indicated that each protocol had a high level of specificity as to the mechanism by which mutations were induced and that the two protocols acted via different and apparently reciprocal mechanisms.

MATERIALS AND METHODS

Cells and media. The cells used in this study were derived from the Syrian hamster melanoma cell line RPMI 3460 (17). These cells, called 2E, were selected for their ability to grow with high levels of BrUra in their DNA (4). The basic culture medium for the cells was Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (DMEM). The FBT medium (12) used in INC mutagenesis experiments was DMEM supplemented with 10 μM 5-fluorodeoxyuridine, an inhibitor of de novo thymidylate biosynthesis (5), plus 30 μM BrdUrd and 30 μM dThd. The FBTdC medium (12) used in REP mutagenesis experiments was DMEM supplemented with 10 μM 5-fluorodeoxyuridine, 10 μM BrdUrd, 10 μM dThd, and 10 μM deoxycytidine (dCyd). For the selection of mutants deficient in HGPRT activity, DMEM was supplemented with 36 μM Sgu. For the selection of cells that expressed HGPRT activity, DMEM was supplemented with 100 μM hypoxanthine and 10 μM azaserine (15, 21); this

medium was called HAS medium. For the selection of cells resistant to ouabain (Oua), DMEM was supplemented with 1 mM Oua.

Protocol for INC mutagenesis. Culture dishes (diameter, 100 mm) containing FBT medium were inoculated with 5×10^5 cells. After 24 h, the dishes were rinsed, and the FBT medium was replaced with DMEM supplemented with 10 μM dThd. This mutagenesis protocol had no effect on the survival of treated cells. The cells were allowed to express mutations in this nonselective medium for either 7 days before the selection of Sgu^r mutants or 4 days before the selection of Oua^r mutants or HAS^r revertants. For the selection of Sgu^r mutants, 100-mm culture dishes containing DMEM supplemented with Sgu were inoculated with 10^5 cells. For the selection of either Oua^r mutants or HAS^r revertants, 100-mm culture dishes containing DMEM supplemented with either Oua or HAS were inoculated with 5×10^5 cells. The plating efficiency of the cells was determined by inoculating 100-mm culture dishes containing DMEM with 10^2 cells. After 9 to 12 days, the dishes to be scored were stained, and colonies of 50 or more cells were counted. The mutation frequencies were expressed as resistant colonies per 10^6 cells, after correction for plating efficiencies in the absence of the selective agent.

Protocol for REP mutagenesis. Culture dishes (diameter, 100 mm) containing FBTdC medium were inoculated with 10^5 cells. After 4 days of growth, during which time the cells were allowed to incorporate BrUra into their DNA under non-mutagenic conditions, the dishes were rinsed, and the FBTdC medium was replaced with DMEM supplemented with 10 μM dThd. After 3 h, to allow for the clearing of all the BrdUTP from the nucleotide pools, the cells were trypsinized, and culture dishes (diameter, 100 mm) containing DMEM supplemented with either 10 μM dThd or 1 mM dThd plus 100 μM dCyd were inoculated with 5×10^5 cells. After 3 days of growth, the cells were trypsinized, and 5×10^5 cells were replated into culture dishes (diameter, 100 mm) containing DMEM to allow for the expression of mutations. This mutagenesis protocol had no effect on the survival of treated cells. The expression and selection of mutants and revertants were performed as described above for the INC mutagenesis protocol.

Protocol for EMS mutagenesis. Culture dishes (diameter, 100 mm) containing DMEM were inoculated with 5×10^5 cells. After 6 h, the medium was brought to a final concentration of 5 mM ethyl methanesulfonate (EMS). After 24 h, the dishes were rinsed and renewed with DMEM. This protocol resulted in approximately 50% survival of the treated cells. The expression and selection of mutants and revertants were performed as described above for the INC mutagenesis protocol.

Protocol for MNNG mutagenesis. Culture dishes (diameter, 100 mm) containing DMEM were inoculated with 2.5×10^5 cells. The next day, the medium was brought to a final concentration of 10 μM *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). After 3 h, the dishes were rinsed and renewed with DMEM. This protocol resulted in approximately 50% survival of the treated cells. The expression and selection of mutants and revertants were performed as described above for the INC mutagenesis protocol.

RESULTS

Induction of Sgu^r mutants by INC mutagenesis. The INC mutagenesis protocol was used to induce 10 independent Sgu^r mutants to be used in the reversion analyses. One Sgu^r clone was isolated from each of 10 independent selections. In these experiments, Sgu^r mutants were induced at an average frequency of 1.5×10^{-3}, whereas uninduced control cultures gave rise to Sgu^r colonies at frequencies of $<1 \times 10^{-6}$. The 10 Sgu^r clones, designated INC-1, -2, etc., were maintained as independent cell lines in DMEM supplemented with Sgu.

Induction of Sgu^r mutants by REP mutagenesis. The REP mutagenesis protocol was used to induce 10 independent Sgu^r mutants to be used in the reversion analyses. One Sgu^r clone was isolated from each of 10 independent selections. In these experiments, Sgu^r mutants were induced at an average frequency of 3.6×10^{-3}, whereas uninduced control cultures gave rise to Sgu^r colonies at frequencies of $<1 \times 10^{-6}$. The 10 Sgu^r clones, designated REP-1, -2, etc., were maintained as independent cell lines in DMEM supplemented with Sgu.

Induction of Sgu^r mutants with chemical mutagenesis. The chemical mutagen EMS was used to induce 14 independent Sgu^r mutants to be used in the reversion analyses. One Sgu^r clone was isolated from each of the independent selections. In these experiments, Sgu^r mutants were induced at an average frequency of 2.5×10^{-3}, whereas uninduced control cultures gave rise to Sgu^r colonies at frequencies of $<1 \times 10^{-6}$. The 14 Sgu^r clones, designated EMS-1, -2, etc., were maintained as independent cell lines in DMEM supplemented with Sgu.

Isolation of spontaneous Sgu^r mutants. In a long series of experiments, eight spontaneous Sgu^r mutants were isolated for use in the reversion analyses. In these experiments, a number of independent cultures of 2E cells were established by inoculating 100-mm culture dishes containing DMEM with 10^4 cells. After 7 days of growth for expression, 10 100-mm culture dishes containing DMEM supplemented with Sgu were inoculated with 10^5 cells for each of the cultures. After 9 days, one Sgu^r clone was isolated from each of the independent selections that were found to give rise to Sgu^r colonies. (A number of the independent selections did not give rise to any Sgu^r colonies.) In these experiments, Sgu^r mutants arose spontaneously at an average frequency of 2.3×10^{-7}. The eight spontaneous Sgu^r clones, designated SPO-1, -2, etc., were maintained as independent cell lines in DMEM supplemented with Sgu.

Reversion analysis. The purpose of isolating the various Sgu^r mutants described above was to attempt to determine the specificity, if any, of the mutagenic mechanisms of INC and REP mutagenesis by analyzing the ability of mutants induced by these protocols to be reverted by them. In the standard reversion assay, each of the independent Sgu^r mutants was tested for (i) spontaneous reversion, (ii) reversion by INC mutagenesis, (iii) reversion by REP mutagenesis, (iv) reversion by EMS mutagenesis, and (v) reversion by MNNG mutagenesis.

Spontaneous reversion or stability of the Sgu^r mutants was determined by growth in the absence of Sgu. After 2 weeks of growth in nonselective medium, each Sgu^r mutant cell line was tested for its ability to plate and grow in HAS medium, a selection system which specifically requires the expression of HGPRT activity (15, 21). The selections for HAS^r revertants were performed as described for INC mutagenesis in Materials and Methods. All other mutagenesis protocols and selection procedures were also performed as described in Materials and Methods.

As a positive control for mutagenesis in the reversion assays, selection for the induction of Oua^r mutants was performed along with the selection of HAS^r revertants.

Reversion of INC mutagenesis-induced Sgu^r mutants. All 10

INC mutants were found to be stable and did not spontaneously revert to HAS^r (Table 1). They were, therefore, all subjected to further analysis. When they were subjected to INC mutagenesis, they were not significantly induced to revert to HAS^r (Table 1). However, when they were subjected to REP mutagenesis, many were found to be efficiently reverted to HAS^r. When they were mutagenized with either EMS or MNNG, the results were similar to those observed with INC mutagenesis, i.e., no significant reversion to HAS^r. All of the mutagenesis protocols gave rise to relatively high and comparable frequencies of Oua^r mutants.

Reversion of REP mutagenesis-induced Sgu^r mutants. All 10 REP mutants were found to be stable and did not spontaneously revert to HAS^r (Table 2). They were, therefore, all subjected to further analysis. When they were subjected to REP mutagenesis, the majority were not significantly induced to revert to HAS^r (Table 2). However, when they were subjected to INC mutagenesis, 5 of the 10 lines were found to be reverted to HAS^r. When the REP mutants were mutagenized with either EMS or MNNG, the results were similar to those observed with INC mutagenesis, i.e., a number of lines were significantly reverted to HAS^r. All of the mutagenesis protocols gave rise to relatively high and comparable frequencies of Oua^r mutants.

Reversion of EMS mutagenesis-induced Sgu^r mutants. Of 14 EMS-induced mutants, 13 were found to be stable and did not spontaneously revert to HAS^r (Table 3). These 13 were, therefore, subjected to further analysis. When they were subjected to INC mutagenesis, they were not significantly induced to revert to HAS^r (Table 3). However, when they were subjected to REP mutagenesis, at least half of the lines were found to be reverted to HAS^r. When the EMS mutants were mutagenized with MNNG, the results were similar to those observed with INC mutagenesis, i.e., 11 of 13 lines were not significantly reverted to HAS^r. The exception to this observation involved two mutant lines (SPO-6 and -7) which were strongly reverted to HAS^r by the action of MNNG but not significantly reverted by the other mutagenesis protocols. All of the mutagenesis protocols gave rise to relatively high and comparable frequencies of Oua^r mutants.

Reversion of spontaneously arising Sgu^r mutants. Analysis of the eight independent spontaneously arising Sgu^r mutant lines resulted in the detection of four distinct groups of mutants. Five of the eight SPO mutants were found to be stable and did not spontaneously revert to HAS^r, whereas three (SPO-3, -4, and -6) were found to revert spontaneously (Table 4). The five stable mutants were, therefore, subjected to further analysis. Of the five, two (SPO-7 and -8) were observed not to revert to HAS^r when subjected to either INC, REP, EMS, or MNNG mutagenesis (Table 4). Of the remaining three, two (SPO-1 and -2) were found to revert by INC, EMS, and MNNG mutagenesis but not by REP mutagenesis, whereas one (SPO-5) was observed to revert by REP mutagenesis but not by either INC, EMS, or MNNG mutagenesis. Again, comparable frequencies of Oua^r mutants were induced in each cell line by the various mutagenesis protocols.

HGPRT activity in mutants and revertants. A representative group of Sgu^r mutants (three from each of the INC, REP, EMS, and SPO groups) were assayed for HGPRT

TABLE 1. Reversion analysis of INC mutagenesis-induced Sgu^r mutants

Cell line	No. of HAS^r revertants/10^6 cells by the following mutagenesis protocol[a]:				
	Spontaneous	INC	REP	EMS	MNNG
INC-1	<1	<2	20	3	1
INC-2	<1	<3	12	<1	<1
INC-3	<1	<3	32	<1	<1
INC-4	<1	<1	72	<1	<1
INC-5	<1	<3	9	<3	<1
INC-6	1	1	183	<2	<1
INC-7	<1	<1	41	<1	1
INC-8	<1	<1	229	<3	<1
INC-9	<1	<1	310	<1	<1
INC-10	<1	2	6	<3	<1
Oua^r[b]	<1	423 ± 300	492 ± 171	271 ± 44	111 ± 49

[a] Details of the mutagenesis protocols are given in Materials and Methods.
[b] Oua^r is expressed as the number of mutants per 10^6 cells after correction for plating efficiency in the absence of selection. The values are shown are the mean ± the standard deviation of the frequencies induced by each mutagenesis protocol in each mutant being assayed for reversion.

TABLE 2. Reversion analysis of REP mutagenesis-induced Sgu^r mutants

Cell line	No. of HAS^r revertants/10^6 cells by the following mutagenesis protocol[a]:				
	Spontaneous	INC	REP	EMS	MNNG
REP-1	<1	190	<2	80	26
REP-2	<1	18	1	62	39
REP-3	<1	110	<2	33	13
REP-4	<1	<1	3	58	5
REP-5	<1	<2	<1	20	28
REP-6	<1	<1	6	33	25
REP-7	<1	16	3	<1	2
REP-8	<1	2	17	<1	<1
REP-9	<1	116	8	56	7
REP-10	<1	<1	2	23	8
Oua^r[b]	<1	354 ± 238	359 ± 230	225 ± 102	161 ± 40

[a] Details of the mutagenesis protocols are given in Materials and Methods.
[b] See footnote[b], Table 1.

TABLE 3. Reversion analysis of EMS mutagenesis-induced Sgu^r mutants

Cell line	No. of HAS^r revertants/10^6 cells by the following mutagenesis protocol[a]:				
	Spontaneous	INC	REP	EMS	MNNG
EMS-1	<1	3	192	ND[b]	<1
EMS-2	<1	6	314	ND	<1
EMS-3	<1	<5	16	ND	<2
EMS-4	<1	<3	<2	ND	<2
EMS-5	244	ND	ND	ND	ND
EMS-6	<1	<8	8	ND	227
EMS-7	<1	<1	<1	ND	225
EMS-8	<1	<2	174	ND	2
EMS-9	<1	<2	14	ND	<1
EMS-10	<1	<1	33	ND	<2
EMS-11	<1	3	166	ND	<4
EMS-12	<1	<2	103	ND	<1
EMS-13	<1	<1	46	ND	<1
EMS-14	<1	12	189	ND	<2
Oua^r[c]	<1	367 ± 161	264 ± 194	ND	356 ± 201

[a] Details of the mutagenesis protocols are given in Materials and Methods.
[b] ND, Not determined.
[c] See footnote *b*, Table 1.

TABLE 4. Reversion analysis of spontaneous Sgu^r mutants

Cell line	No. of HAS^r revertants/10^6 cells by the following mutagenesis protocols[a]:				
	Spontaneous	INC	REP	EMS	MNNG
SPO-1	1	22	1	167	26
SPO-2	<1	21	1	65	53
SPO-3	14	ND[b]	ND	ND	ND
SPO-4	20	ND	ND	ND	ND
SPO-5	1	<1	21	<1	<1
SPO-6	45	ND	ND	ND	ND
SPO-7	<1	<1	<1	<1	<1
SPO-8	<1	<1	<1	<1	<1
Oua^r[c]	<1	381 ± 246	252 ± 132	558 ± 149	153 ± 59

[a] Details of the mutagenesis protocols are given in Materials and Methods.
[b] ND, Not determined.
[c] See footnote *b*, Table 1.

activity in vitro, by the method of Olsen and Milman (18). It was found that all of the mutants had <0.5% of the activity of the wild-type 2E cells (data not shown). When revertants of these mutants were tested, it was found that they had regained levels of HGPRT activity (70 to 100%) comparable to that of the wild-type 2E cells (data not shown).

DISCUSSION

This report has described a genetic analysis designed to compare the mutagenic specificities of two different mutagenesis protocols, both of which utilize the dThd analog, BrdUrd, as a mutagen. The assay involved the induction of mutants resistant to the toxic effects of Sgu by both protocols and subsequent testing to determine the specificity of reversion of these mutants by these same mutagenesis protocols. In all of the reversion assays, the level of Oua^r mutants induced was monitored to assure that the cells being tested were being subjected to an approximately equal mutagenic load. (It should be noted that the mutation frequencies obtained in these experiments were relatively high when compared with frequencies obtained in similar experiments reported in the literature. This may have been due to the use of Syrian hamster cells in these experiments. However, we have obtained results similar to those presented here with Chinese hamster cells, albeit at lower frequencies of forward and reverse mutation.) The hypothesis to be tested was that if INC and REP mutagenesis acted via different mechanisms, and possibly produced different base substitutions, then mutants induced by each protocol would have different responses when assayed for reversion induced by various mutagenesis protocols.

The results obtained indicated that the two BrdUrd mutagenesis protocols acted via reciprocal mechanisms with a high degree of specificity, i.e., the mutants induced by INC mutagenesis were much more efficiently induced to revert by REP mutagenesis than by INC mutagenesis, and the mutants induced by REP mutagenesis were more efficiently reverted by INC mutagenesis than by REP mutagenesis. The very low levels of reversion, if any, observed when mutants were tested for reversion by the mutagenesis protocol by which they were originally induced suggested that the revertants that were observed were probably not the result of second-site reversion. A clear explanation for the apparent lack of significant levels of second-site reversion, which others have observed in mutants at this locus (9), is unavailable at this time and will require further investigation. These findings strongly support the above hypothesis.

Analysis of spontaneously arising mutants resulted in some interesting observations. Although most of the spontaneous mutants either had a high rate of spontaneous reversion or did not revert at all, the stable mutants that could be induced to revert did so with a high degree of specificity. Of three mutant cell lines, two were reverted by INC, EMS, and MNNG mutagenesis but not by REP mutagenesis, whereas the remaining mutant cell line was reverted only by REP mutagenesis and not by either INC, EMS, or MNNG mutagenesis. These results again indicated a high degree of specificity for the mechanisms of the BrdUrd mutagenesis protocols and again suggested that second-site reversion was not a significant factor in these analyses.

To further test the hypothesis, two alkylating agents, EMS and MNNG, were used as mutagens. It was found that they had a high degree of mutagenic specificity when tested by the reversion assays, i.e., (i) both EMS and MNNG were effective in reverting REP-induced but not INC-induced mutants, and (ii) the majority of EMS-induced mutants were reverted by REP mutagenesis and not by either INC or MNNG mutagenesis. Previous determinations of mutagenic specificity for EMS and MNNG in bacteria (6) and yeast cells (19) showed a strong preference for G · C-to-A · T transitions. If EMS and MNNG had a similar preference in mammalian cells, then the results of the reversion analysis suggested that INC mutagenesis, which appeared to act with a mechanism similar to that of EMS and MNNG mutagenesis, induced G · C-to-A · T transitions. Because REP mutagenesis appeared to act via a mechanism different from and reciprocal to the mechanisms of INC, EMS, and MNNG mutagenesis, the results were consistent with the suggestion that REP mutagenesis induced A · T-to-G · C transitions. It should be noted that these same base transitions were proposed by Freese (10) for his model for the mutagenic action of BrUra.

BrdUrd mutagenesis in mammalian cells appears to be unique in that by changing the experimental protocol, one can induce highly specific but different mutagenic events with a single mutagen. The mutagenesis protocols described in this report may prove to be useful in generating mutations with specific base changes in mammalian cells.

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