Specificity of base substitutions induced by the acridine mutagen ICR-191: Mispairing by guanine N7 adducts as a mutagenic mechanism

ARTICLE *in* GENETICS · JANUARY 1992

Impact Factor: 5.96 · Source: PubMed

3 AUTHORS, INCLUDING:

Sudhir Sahasrabudhe
Rines

33 PUBLICATIONS **3,943** CITATIONS

SEE PROFILE

Miyama-Inaba, M., Ohno, T., Inaba, K., Ajisaka, K., Suzuki, R., Kumagai, K., & Masuda, T. (1987) *Immunology 61*, 43–50.
Morawetz, H. (1975) *Macromolecules in Solution*, pp 189–192, Wiley and Sons, Inc., New York.
Morel, G. A., Yarmush, D. M., Colton, C. K., Benjamin, D. C., & Yarmush, M. L. (1988) *Mol. Immunol. 25*, 7–16.
Moyle, W. R., Lin, C., Corson, R. L., & Ehrlich, P. H. (1983) *Mol. Immunol. 20*, 439–482.
Murphy, R. M. (1989) Antigen–Antibody Complexes: Size, Structure and Reactivity with Protein A. Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, MA.
Murphy, R. M., Slayter, H., Schurtenberger, P., Chamberlin, R. A., Colton, C. K., & Yarmush, M. L. (1988) *Biophys. J. 54*, 45–56.
Reisberg, M. A., & Rossen, R. D. (1981) *Clin. Exp. Immunol. 46*, 443–452.
Schmidt, M., & Stockmayer, W. H. (1984) *Macromolecules 17*, 509–514.
Schumaker, B. N., Green, G., & Wilder, R. L. (1973) *Immunochemistry 10*, 521–528.
Schumaker, V. N., Seegan, G. W., Smith, C. A., Ma, S. K., Rodwell, J. D., & Schumaker, M. F. (1980) *Mol. Immunol. 17*, 413–423.
Schweitzer-Stenner, R., Licht, A., Luscher, I., & Pecht, I. (1987) *Biochemistry 26*, 3602–3612.
Segal, D. N., Dower, S. K., & Titus, J. A. (1983) *J. Immunol. 130*, 130–137.
Seinfeld, J. H., & Lapidus, L. (1974) *Mathematical Methods in Chemical Engineering. Vol. 3. Process Modeling, Estimation and Identification*, Prentice-Hall, Englewood Cliffs, NJ.
Sharp, P., & Bloomfield, V. A. (1968) *Biopolymers 6*, 1201–1211.
Steensgard, J., Liu, B. M., Cline, G. B., & Moller, N. P. H. (1977) *Immunology 32*, 445–456.
Steensgard, J., Jacobson, C., Lowe, J., Ling, N. R., & Jefferis, R. (1982) *Immunology 46*, 751–760.
Wofsy, C., & Goldstein, B. (1987) *Mol. Immunol. 24*, 151–161.
Wofsy, C., Goldstein, B., & Dembo, M. (1978) *J. Immunol. 121*, 593–601.
Yamakawa, H., & Fujii, M. (1973) *Macromolecules 6*, 407–415.
Yarmush, D. M., Morel, G., & Yarmush, M. L. (1987) *J. Biochem. Biophys. Methods 14*, 279–289.
Yarmush, D. M., Murphy, R. M., Colton, C. K., Fisch, M., & Yarmush, M. L. (1988) *Mol. Immunol. 25*, 17–25.

Induction of G·C to A·T Transitions by the Acridine Half-Mustard ICR-191 Supports a Mispairing Mechanism for Mutagenesis by Some Bulky Mutagens†

Sudhir R. Sahasrabudhe, Xun Luo, and M. Zafri Humayun*
Department of Microbiology and Molecular Genetics, University of Medicine and Dentistry of New Jersey–New Jersey Medical School, 185 South Orange Avenue, MSB F607, Newark, New Jersey 07103-2757
Received March 15, 1990; Revised Manuscript Received August 22, 1990

ABSTRACT: As the most nucleophilic atom in DNA, the guanine N7 atom is a major site of attack for a large number of chemical mutagens as well as chemotherapeutic agents. Paradoxically, while methylation of guanine N7 is believed to be largely nonmutagenic, aflatoxin B_1, among the most potent mutagens, appears to exert its mutagenic activity through adduction at this site. On the basis of an analysis of the specificity of mutations induced by various adduct forms of aflatoxin B_1, we have previously proposed mechanisms that can both resolve the paradox and account for the specificity of mutagenesis by aflatoxin B_1. The hypothesized mechanisms specify how a bulky guanine N7 lesion can promote G·C to A·T transitions as well as frame-shift mutations. Since the proposed mechanisms are in principle lesion-independent, a simple test of the proposed mechanisms would be to examine the specificity of mutations induced by a structurally different bulky guanine N7 adduct. Toward this goal, M13 replicative form DNA was subjected to in vitro adduction with the acridine mutagen ICR-191 and transfected into *Escherichia coli*. Mutations in the LacZ(α) gene segments were scored and defined at the sequence level. The results show that ICR-191 adduction induces both base substitutions and frame shifts with near-equal efficiency. A clear majority of base substitutions were G·C to A·T transitions. On the other hand, unlike aflatoxin B_1 which could induce both −1 and +1 frameshifts, ICR-191 appears to predominantly induce +1 frame shifts. This preference appears to arise by lesion-dependent mechanisms. The data presented in this communication are consistent with the proposed mechanisms for base substitution mutagenesis by bulky guanine N7 lesions and permit a further refinement of the previously proposed mechanisms for frame-shift induction by bulky DNA lesions.

Alkylating agents, which encompass a very broad range of laboratory mutagens and natural and man-made carcinogens as well as chemotherapeutic agents, are known to react with DNA predominantly at the guanine N7 position. On the other hand, the role of guanine N7 adduction in mutagenesis and carcinogenesis is poorly understood. For a long time, it has been thought that guanine N7 adduction may represent "tolerated" DNA damage which is neither lethal nor mutagenic, leading to a search for minor premutagenic lesions such as *O*-alkylated bases or lethal lesions such as cross-links. Experimental evidence on several O-alkylated bases has established that these lesions are indeed mutagenic [for brief reviews, see Basu and Essigmann (1988) and Walker (1984)].

† This work was supported by USPHS Grant CA27735.
* Corresponding author.

0006-2960/90/0429-10899$02.50/0

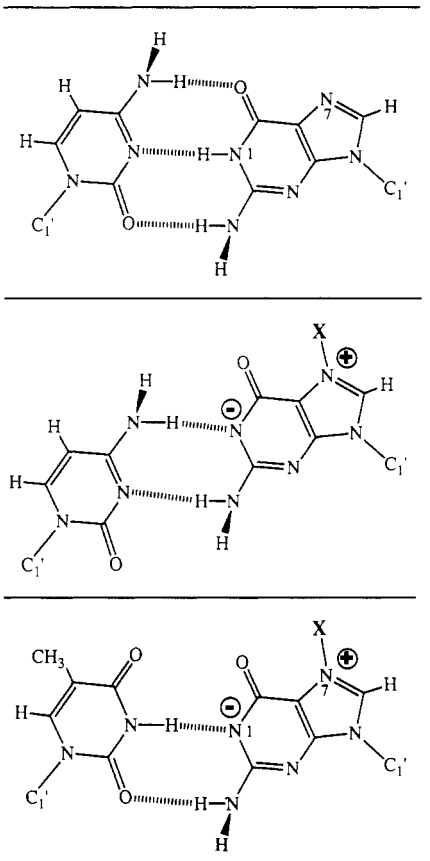

FIGURE 1: Proposed mispairing mechanisms for N7-adducted guanine [after Lawley and Brookes (1961)]. Top panel, a Watson–Crick C·G bp (note hydrogen bond at N1); center, non-Watson–Crick base pair between C and N1-deprotonated G; bottom, mispairing between T and N1-deprotonated G. Note that deprotonation (zwitterion formation) is a consequence of the positive charge in the imidazole ring and can be abolished by imidazole ring opening. X denotes a bulky adduct.

That guanine N7 adduction can be mutagenic is strongly supported by information that has recently become available on the mechanisms of mutagenesis by the fungal metabolite aflatoxin B_1 (AFB1).[1] AFB1, among the most powerful mutagens in bacterial assays (McCann et al., 1975), is believed to react almost exclusively with the guanine N7 position, and this reaction is believed to be responsible for the toxic, mutagenic, and carcinogenic properties of this mutagen (Busby & Wogan, 1984).

We have recently examined the specificity of base changes induced by AFB1 under a variety of experimental conditions in the *Escherichia coli*–M13 system (Refolo et al., 1987; Sambamurti et al., 1988; Sahasrabudhe et al., 1989). Our data on the differences in the specificity of base substitutions induced by the primary guanine N7–AFB1 lesions and that by the the secondary AFB1 (guanine imidazole ring opened) lesions have raised the possibility that guanine N7 adducts might promote mutagenesis by acting as noninstructional as well as mispairing lesions. The proposed mispairing is mediated by deprotonation at the guanine N1 position provoked by N7 adduction (Lawley & Brookes, 1961). More specifically, N1 deprotonation creates the potential for G·T mispairing, resulting in G·C to A·T transitions (Figure 1). Since N1 deprotonation would be favored when DNA is in single-stranded conformation, the ability of a DNA lesion to transiently block replication was proposed to be crucial. This hypothesis could also account for the relative nonmutagenicity of N7 adduction by methylating agents, because such lesions are not believed to block replication (Larson et al., 1985) and are therefore replicated before significant N1 deprotonation. The ability of a lesion to block replication may also play a central role in promoting frame-shift mutations, as considered briefly under Discussion and more extensively elsewhere (Refolo et al., 1987).

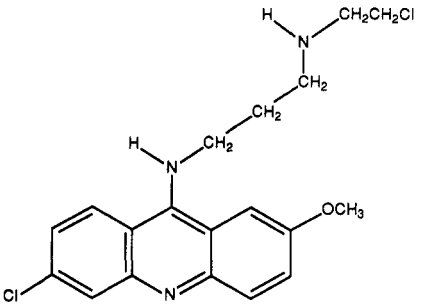

FIGURE 2: Structure of ICR-191.

Since the hypothesis does not assign a specific role to the chemical nature of the lesion but rather ascribes significance to guanine N7 adduction and to the ability of the adduct to transiently block replication, it should apply to other bulky lesions at the guanine N7 position. Therefore, one simple test of this hypothesis would be to examine the specificity of mutations induced by a structurally different bulky adduct at the guanine N7 position. Specifically, the hypothesis predicts that G·C to A·T transitions should be promoted by such bulky mutagens. On the other hand, if bulky lesions were processed as noninstructional lesions bypassed by a misincorporation of adenine [the "A rule"; e.g., Rabkin and Strauss (1984)], then G·C to T·A transversions should predominate.

ICR-191 is a well-known laboratory mutagen which consists of an acridine moiety attached to a side arm terminating in a half-mustard (Figure 2). Like AFB1, ICR-191 is a versatile mutagen capable of inducing both frame-shift and base substitution mutations in the Ames bacterial reversion assay (McCann et al., 1975). ICR-191 is expected to react with DNA with the specificity expected for other nitrogen mustards, with reaction at the guanine N7 atom predominating (Mattes et al., 1986). In addition, the acridine core of ICR-191 is by itself known to be an efficient DNA intercalator. Acridines underivatized by the addition of an alkylating side chain are by themselves frame-shift mutagens [e.g., Skopek and Hutchinson (1984) and Striesinger and Owen (1985)]. Mutations detected in forward assays in which *cells are exposed to ICR-191* should therefore have a dual source: those deriving from the noncovalent DNA binding (presumably intercalation) and those deriving from covalent binding (with or without concomitant intercalation) by the mutagen.

Since we are specifically interested in the mutagenic mechanisms of covalent adducts, it is necessary to reduce or eliminate the mutagenic contribution of noncovalently bound ICR-191. Here, we describe an analysis of the specificity of forward mutations induced by in vitro adduction of phage M13 double-stranded replicative form (RF) DNA upon transfection into *E. coli*.

Materials and Methods

Strains, Media, and Chemicals. *E. coli* strain KH2AM ($uvrA^-/mucAB^+$) and the phage M13 derivative AB28 have been described elsewhere (Sambamurti et al., 1988). Media and reagents for transfection experiments were as described

[1] Abbreviations: AFB1, aflatoxin B_1; ICR-191, 6-chloro-9-[[3-[*N*-(2-chloroethyl)amino]propyl]amino]-2-methoxyacridine; RF DNA, replicative form DNA.

(Sambamurti et al., 1988). 6-Chloro-9-[[3-[*N*-(2-chloroethyl)amino]propyl]amino]-2-methoxyacridine dihydrochloride, more commonly known as the acridine mutagen ICR-191, was from Terochem (Edmonton, Alberta) or Sigma. For the purpose of spectrophotometric quantitation of ICR-191, an ϵ^{276} value of 45 600 was used.

DNA Preparation and ICR-191 Treatment. For the preparation of M13AB28 RF DNA, *E. coli* KH2A (Sambamurti et al., 1988) cells were grown at 37 °C in a rich medium (YT) to a density of (2–4) × 10^8 cells/mL and infected with phage M13AB28 at a multiplicity of 4–10. After 45 min at 37 °C with vigorous aeration, chloramphenicol was added to a final concentration of 50–100 μg/mL, and incubation at 37 °C with aeration was continued for an additional 45 min. The RF DNA was extracted from the cell pellet by the "alkaline lysis" protocol followed by purification of form I DNA on cesium chloride/ethidium bromide gradients according to standard procedures described in a laboratory manual (Heilig, 1989). The RF DNA was modified with ICR-191 by the following procedures. DNA (50 μg) was incubated in the dark at room temperature with 0.166–138.5 μM ICR-191 (delivered from a freshly prepared aqueous stock solution) in 400 μL of 20 mM Hepes, pH 6.8, in a 1.5-mL polypropylene Eppendorf test tube. The DNA was recovered by mixing in 45 μL of 3 M sodium acetate (pH 6.8) followed by 800 μL of cold absolute ethanol. The tube was cooled in an ethanol/dry ice bath for 15 min and centrifuged for 30 min at 15000*g*. The pellet was resuspended in 200 μL of 20 mM Hepes (pH 6.8) and the DNA recovered by two more rounds of ethanol precipitation as described above. The final DNA pellet was dried under reduced pressure in a Savant Speed-Vac machine and resuspended in 50 μL of 10 mM Hepes (pH 6.8). Mock-treated DNA was subjected to identical treatment except for omitting ICR-191 from the reaction.

Alkali-Labile Site Analysis. Plasmid pUC8 (Vieira & Messing, 1982) which contains the same LacZ(α) sequence as M13AB28 was prepared by standard "alkaline lysis" procedures (Heilig, 1989) and purified over cesium chloride/ethidium bromide gradients. The plasmid DNA (20 μg) was digested with 40 units each of *Eco*R1 and *Bgl*1 (New England Biolabs) in 100 μL of buffer (150 mM KCl, 10 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, and 1 mg/mL bovine serum albumin) for 60 min at 37 °C. The digestion was terminated by extracting the reaction mix once with phenol/chloroform (1:1) and three times with ether and the DNA recovered by standard ethanol precipitation procedures. *Eco*R1–*Bgl*1 digestion of pUC8 yields three fragments: a short fragment (*Eco*R1–*Bgl*1 188 bp) from a part of the LacZ(α) sequence and two much larger fragments from the remainder of the plasmid genome (*Bgl*1–*Bgl*1 1118 bp and *Eco*R1–*Bgl*1 1380 bp). The *Eco*R1 end (which has a recessed 3′-OH terminus) was specifically labeled by an end-filling reaction with 8 units of *E. coli* DNA polymerase I (Klenow fragment; New England Biolabs) and [α^{32}P]dATP (3000 Ci/mmol; Amersham) in 80 μL of buffer (10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 7.5 mM dithiothreitol) for 30 min at 37 °C. The reaction was chased by adding 1 μL of 1 mM dATP and an additional 2 units of the polymerase and continued incubation at 37 °C for an additional 15 min. The reaction mix was set on ice, the DNA was freed from small molecules by a Sephadex G-25 "spun column" and fractionated on a 5% polyacrylamide gel. The 188 bp fragment was identified by autoradiography, extracted from the gel by diffusion using standard procedures, and purified by ethanol precipitation. The DNA pellet was resuspended in 100 μL of water and kept frozen until use. This procedure yielded DNA containing a total of 5–7 million cpm. Labeled DNA (100 000 cpm) was mixed with 2 μg of carrier DNA (unlabeled sonicated calf thymus DNA) and reacted with ICR-191 (0–100 μM) in 200 μL of 50 mM Hepes (pH 6.8) for 60 min at room temperature. The reaction was terminated by adding 50 μL of a "DMS stop" solution (1.5 M sodium acetate, pH 7, 1 M 2-mercaptoethanol, and 100 μg/mL tRNA). Subsequent steps for DNA recovery, piperidine cleavage, and sequence gel analysis were carried out as described in a laboratory handbook (Maniatis et al., 1982).

Transfection, Mutant Identification, and DNA Sequencing. Procedures for transfection of mock-treated and ICR-191-treated RF DNA into *E. coli* KH2AM cells and methods for identification of LacZ(α)$^-$ AB28 phage progeny and for DNA sequencing have been described (Sambamurti et al., 1988).

Results

The experimental system, described elsewhere (Sambamurti et al., 1988), consists of (a) in vitro adduction of M13 RF DNA with ICR-191, (b) transfection into *E. coli*, (c) scoring and confirmation of mutations in the LacZ(α) gene, and (d) DNA sequence analysis of LacZ(α) mutants. The specific M13 phage used (AB28) is derived from M13mp2 and M13mp8 and can grow in Sup^0 *E. coli* hosts to yield dark blue plaques. LacZ(α)$^-$ phage plaques are identified by colorless or light blue phenotype which is confirmed as described (Kunkel, 1984). All of the experiments described here were carried out in *E. coli* KH2AM, and excision-deficient (uvrA$^-$) strain bearing the mucAB$^+$ plasmid pGW270 (Sambamurti et al., 1988). A similar *Salmonella* strain had been previously used by Ames and co-workers to demonstrate that ICR-191 could revert base substitution as well as frame-shift mutations (McCann et al., 1975).

Table I shows the effect of in vitro treatment of M13AB28 RF DNA with ICR-191 on phage survival and on mutation frequency in the LacZ(α) gene segment. In comparison to mock-treated DNA (top row, Table I), there is a dose-dependent drop in survival, and a concomitant increase in mutation frequency, with both effects leveling off around an initial ICR-191 concentration of 55–83 μM. Survival in UV-(SOS)-induced cells ("+SOS") does not appear to be markedly different from that in unirradiated ("–SOS") cells. Mutation frequencies induced by ICR-191 in unirradiated and in UV-irradiated cells appear to be similar at low and high ICR-191 concentrations, but different at intermediate concentrations. In unirradiated (–SOS) cells, the mutation frequency increases to about 10-fold in the dose range of 0–1.39 μM ICR-191 and remains at this level up until a dose of 55.4 μM, but reaches a second plateau of about 19-fold above the background at or above the ICR-191 concentration of 83.11 μM. In UV-irradiated cells, the increase in mutation frequency is gradual until it begins to level off at about 19-fold above background around 83 μM ICR-191. Therefore, even though the mutation frequencies at low and high doses are similar in unirradiated and irradiated cells, the mutation frequencies are up to 1.5-fold higher in UV-irradiated cells at the intermediate doses. For an analysis of the specificity of induced mutations, we used an initial ICR-191 concentration of 13.85 μM. At this level of treatment, mutation frequency is increased 11-fold in unirradiated cells and 15-fold in UV-irradiated cells.

Table II presents a summary of the sequence analysis of 119 LacZ(α)$^-$ mutants obtained from transfection of ICR-191-treated RF DNA into unirradiated cells and of 67 similar mutants obtained from irradiated cells. The target sequence for analysis consists of the amino-terminal 300 bp of the LacZ(α) gene segment, extending from base 6217 through

Table I: Effect of ICR-191 Adduction on Survival of Phage M13 and on Mutagenesis in the LacZ(α) Gene[a]

initial ICR-191 concentration[b] (μM)	survival [PFU/μg[c] × 10^5 (%)]		mutation frequency [×10^{-4} (SD)[f]]	
	−SOS[d]	+SOS[e]	−SOS	+SOS
0	4.65 (100)	6.33 (100)	1.00 (0.06)	1.07 (0.26)
0.139	4.77 (103)	5.89 (93)	4.19 (0.47)	4.77 (0.25)
0.69	4.37 (94)	4.8 (76)	4.98 (0.58)	5.07 (0.22)
1.39	4.39 (94)	4.85 (77)	9.24 (1.75)	11.61 (0.34)
6.93	2.06 (44)	2.87 (45)	9.53 (1.36)	13.78 (0.61)
13.85	**1.49 (32)**	**2.09 (33)**	**11.30 (0.92)**	**16.50 (1.01)**
27.70	1.03 (22)	1.79 (28)	10.28 (1.21)	18.87 (0.24)
55.40	0.72 (15)	1.18 (19)	12.36 (1.48)	18.93 (1.47)
83.11	0.62 (13)	1.2 (19)	19.03 (0.09)	21.00 (2.42)
110.81	0.44 (9)	0.72 (11)	19.50 (1.93)	22.81 (1.64)
138.51	0.42 (9)	0.7 (11)	19.52 (0.63)	20.27 (0.77)

[a] Values shown are averages of at least three independent experiments. The boldfaced row represents the conditions under which mutants were isolated for DNA sequence analysis. [b] See Materials and Methods for treatment and DNA recovery procedures. [c] Survival is expressed as plaque-forming units per microgram of transfected DNA. The parenthetical figures are survival values expressed as percentages of those for mock-treated DNA. [d] −SOS denotes experiments in which transfection was carried out in unirradiated *E. coli* KH2AM cells. [e] +SOS denotes experiments in which transfection was carried out in UV-irradiated *E. coli* KH2AM cells (10 J/m^2; Sambamurti et al., 1988). [f] Standard deviation.

Table II: Summary of DNA Sequence Changes Induced by ICR-191 in the M13AB28 LacZ(α) Gene

	−SOS	+SOS	total
(A) Mutation Frequency (×10^{-4})			
mock-treated DNA	1.00	1.07	NA
ICR-191-treated DNA	11.3	16.5	NA
increase: treated/mock (x-fold)	11.3	15.4	NA
(B) Sequence Analysis Summary			
no. sequenced	119	67	186
no. with sequence change[a]	74	30	104
(C) Major Classes of Mutations			
point mutations[b]	57	30	87
126 bp deletions[c]	13	0	13
large deletions[d]	2	0	2
short deletions[e]	1	0	1
short duplications[e]	1	0	1
(D) Frame-Shift Mutations			
all frame shifts	32	16	48
+1	30	14	44
−1	2	2	4
(E) Substitutions			
all substitutions	25	14	39
G·C targeted	19	11	30
G·C to A·T	12	8	20
G·C to T·A	7	3	10
G·C to C·G	0	0	0
A·T targetes	6	3	9
A·T to G·C	5	1	6
A·T to C·G	0	2	2
A·T to T·A	1	0	1

[a] Number of phenotypically LacZ(α)$^-$ mutants showing DNA sequence changes within the sequencing target shown in Figure 3. [b] Point mutations are base substitutions and frame shifts. [c] For an explanation of 126 bp deletions, see text. [d] Large deletions have presumably lost the *E. coli* DNA insert in M13, since the primers used for sequencing the target region showed no evidence of priming these mutant DNAs. [e] See Figure 3 for the sequence changes in the short deletion and in the short duplication.

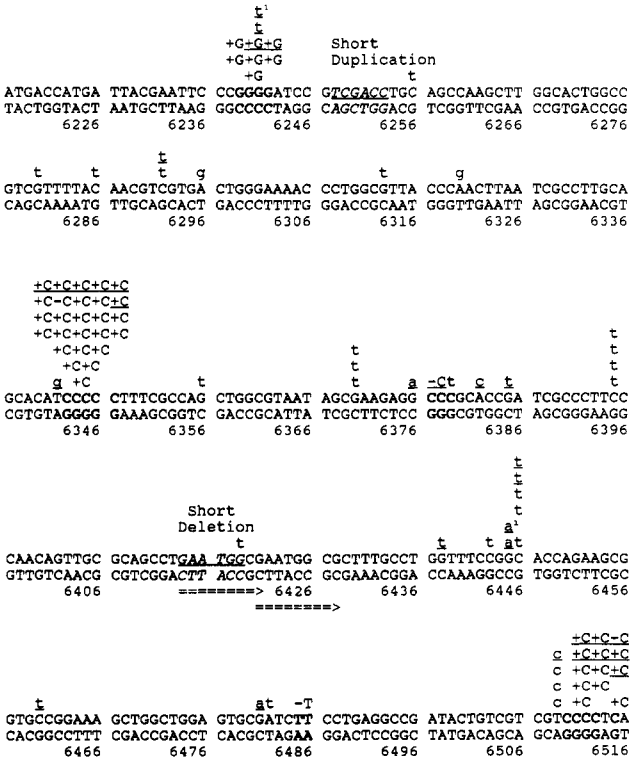

FIGURE 3: A part of the M13AB28 LacZ(α) gene sequence showing base changes induced by ICR-191 in unirradiated and UV-irradiated cells. The sense strand (upper strand) begins at an ATG initiating triplet. Base changes in ICR-191 induced mutations, arbitrarily depicted as changes in the upper strand, are shown above the sequence according to the following conventions: (a) base substitutions are shown in lower case letters above the affected wild-type base; (b) bases added or deleted are indicated by an upper case letter (preceded by a "+" for +1 frame shifts (e.g., +G) or by a "−" for −1 frame shifts (e.g., −C); in addition, the base or base run affected are shown in bold face; (c) the same conventions are used for mutants isolated from unirradiated (−SOS) cells and UV-irradiated (+SOS) cells, except that those from the +SOS conditions are underlined. One mutant showed two base substitutions (t^1 at G6241 and a^1 at G6445). Bases affected in the "short duplication" (top row of sequence) and in the "short deletion" (fourth row from top) are underlined and italicized. In the short deletion, one member of a partly overlapping direct repeat (shown by two rightward arrows under the sequence) is lost.

6516 (Figure 3). (M13mp phages have a 789 nt long *E. coli* DNA insert consisting of a part of the carboxy-terminal end of the LacI gene, the Lac operator/promoter sequence, and codons for the first 145 amino acids of the LacZ gene.) Table IIB,C shows that point mutations are observed within the target sequence in 45% of the −SOS or +SOS mutants. Table IIC shows that while all of the +SOS mutants are point mutations, the −SOS mutations consist of point mutations (77%), "126 bp deletions" (18%), and other mutations (5%). The 126 bp deletions are analogous to the 93 bp deletions previously known for M13mp2 phage and are believed to arise by recombination between the phage LacZ(α) gene and the truncated LacZ gene carried in the host on an F episome (Kunkel, 1984; LeClerc et al., 1984). The polylinker in M13AB28 adds 33 bp to the size of the deletion. The 126 bp deletions, found only among the −SOS mutants, may represent background mutations. However, we have previously observed that 126 bp deletions constitute about 10% of the background

mutations in this system (K. Sambamurti and M. Z. Humayun, unpublished results). Since the background mutation frequency is around 10^{-4}, the relative mutation frequency for background 126 bp deletions is around 10^{-5}. The corresponding relative mutation frequency for ICR-191-treated DNA, from Table II, is 1.23×10^{-4} [fraction of 126 bp deletions (13/119) multiplied by the mutation frequency (11.3×10^{-4})]. Therefore, it is possible that 126 bp deletions are a consequence of reduced lesion bypass in −SOS conditions. According to this notion, stalled replication forks on the phage genome may promote recombination with the F episome by providing appropriate DNA substrates.

Figure 3 depicts a part of the M13AB28 LacZ(α) gene and shows the sequence changes associated with the point mutations induced by ICR-191. The "target sequence" shown in Figure 3 spans bases 6217–6516 and covers the first 100 codons of the LacZ(α) gene segment, including the codons contributed by the polylinker in AB28. Base changes are arbitrarily shown as changes in the plus (upper) strand. Bases gained or lost in frame-shift mutations are represented above the sequence by an upper case letter preceded by the symbol "+" or "−", whereas base substitutions are depicted in lower case letters above the wild-type base. Mutations observed in UV-irradiated (+SOS) cells, similarly depicted, are *underlined*. In addition, a short duplication (*TCGACC*, bases 6248–6253) and a short deletion at a direct repeat (*GAATGG*, bases 6414–6419), both found in −SOS cells, are also shown. Although the number of mutations of this class is low, they have not been previously observed in this experimental system, and it is possible that these are ICR-191 induced.

Table IID,E shows the breakdown of the types of point mutations induced. For both −SOS and +SOS conditions, base substitution mutations constitute about 45% of the point mutations, while the remaining are frame-shift mutations. Two striking features of ICR-191-induced frame shifts are apparent: +1 frame shifts predominate (Table IID); three hot spots (Figure 3; bases 6239–6242, 6343–6347, and 6510–6513) account for virtually all of the frame shifts.

Base substitutions are distributed over a much larger number of sites as compared to frame shifts, with a number of potential hot spots at bases 6370, 6395, 6446, and 6509 (Figure 3). A clear majority of the base substitutions induced by ICR-191 (over 75%) are targeted to G·C bp. It is apparent from Table IIE that the predominant substitution event is a G·C to A·T transition (about 67% of G·C-targeted events, or 50% of all substitutions), while the remaining are G·C to T·A transversions. Most of the A·T-targeted events are A·T to G·C transitions. At the major A·T-targeted substitution hot spot (Figure 3, base 6509), the base change (to a G·C bp) has the effect of extending the adjacent G·C bp run, raising the question whether such mutations are templated by an adjacent base ("duplicative substitutions"; Refolo et al., 1987; Kunkel & Soni, 1988).

Figure 4 shows that in vitro ICR-191 treatment of a restriction fragment derived from the LacZ(α) gene segment results in the induction of alkali-labile sites at guanines, as expected for a guanine N7 alkylating agent. These data also indicate that ICR-191 might preferentially attack guanines occurring in runs as compared to isolated guanines.

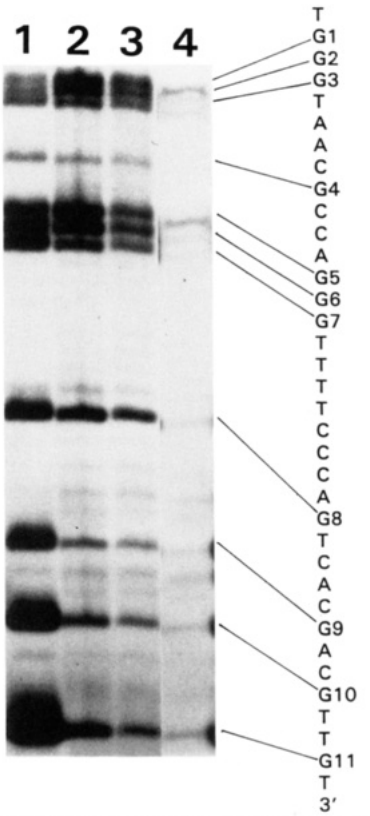

FIGURE 4: Alkali-labile site analysis of an ICR-191-treated 188 bp DNA fragment obtained from the LacZ(α) gene of plasmid pUC8, which contains the same LacZ(α) DNA sequence as M13AB28 (Sambamurti et al., 1988). See Materials and Methods for procedures. The autoradiograph depicts alkali-labile sites occurring in a 34 bp segment of the LacZ(α) complementary strand (bottom strand in Figure 3). The corresponding DNA sequence is shown to the right. Lane 1, DNA subjected to standard Maxam–Gilbert guanine-specific degradation (Maniatis et al., 1982); lane 2, DNA treated with 100 μM ICR-191; lane 3, DNA treated with 10 μM ICR-191; and lane 4, mock-treated DNA. The products were fractionated on a 20% polyacrylamide–8 M urea gel. Preferential attack of guanines in a run can be observed by comparing the relative intensities of the "lone" guanine G4 with those in the runs G1–G3 and G5–G7.

Discussion

Mutagenic Properties of ICR-191. As considered in the introduction, ICR-191 is an interesting model mutagen with two DNA-interactive components: the intercalatable acridine ring system and the alkylating arm constituting a half-mustard. Perhaps the different modes of DNA interaction possible for ICR-191 can account for a seeming contradiction in the mutagenic specificity deduced from bacterial reversion assays and forward assays. In the Ames reversion assay (McCann et al., 1975), ICR-191 is equipotent in inducing frame shifts and base substitutions. In forward assays (Calos & Miller, 1981; Skopek & Hutchinson, 1984) in which bacterial cells are exposed to ICR-191, frame shifts appear to be almost exclusively induced. Since the acridine moiety of ICR-191 is by itself a strong frame-shift mutagen, and since a major DNA interaction of ICR-191 (when cells are treated) might be simple intercalation without covalent binding, forward assays should detect ICR-191 mutagenesis by simple noncovalent as well as by covalent binding. As a result, any induced base substitutions (caused by covalent DNA damage) may be masked by frame shifts (caused by simple intercalation as well as by DNA damage) in forward assays but are uncovered in reversion assays. Since the current approach should detect mutations predominantly from the covalent adduct, the observation of both substitutions and frame shifts is consistent with the above possibility.

Frame-Shift Mutations Induced by ICR-191. Refolo et al. (1987) have previously proposed that the ability of a mutagen to stall replication was a crucial feature of frame-shift mutagens. The ability to stop replication can in principle be mediated by covalent or noncovalent binding, or through an

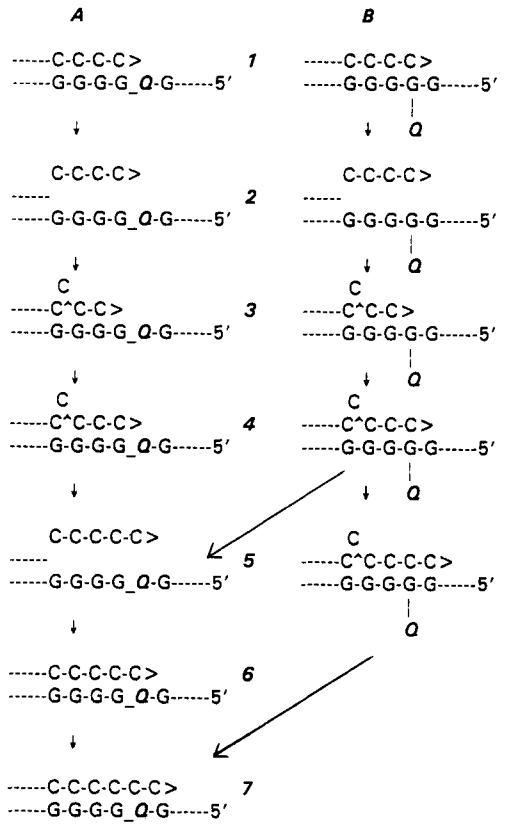

FIGURE 5: Possible mechanisms for +1 frame shifts induced by ICR-191 at a hot spot. Two parallel models, one with the acridine moiety (Q) initially stacked 5′ of the adducted guanine (A) or in an "outside" configuration (B), are depicted. Structures A.1 and B.1 depict stalled replication, which provides an opportunity for denaturation of the primer terminus (A.2 and B.2) followed by primer misalignment (A.3 and B.3). In pathway A, incorporation of an additional base (A.4) and subsequent realignment generate a structure (A.6 and A.7) in which the stacked acridine takes the approximate position of a template base. Continued elongation of structures A.6 or B.5 yields a +1 frame shift. As indicated by the diagonal arrows, structures B.4 and B.5 can rearrange to structures A.6 and A.7.

intermediate such as a protein. Stalling was proposed to open a "time window" during replication for slippage events in the vicinity of the lesion. Since slippage is favored at base runs, a powerful frame-shift mutagen should have the following attributes: (a) ability to transiently block replication; (b) damage preference for base runs. This hypothesis, which neither requires nor prohibits DNA intercalation, ascribes an active role to the DNA lesion in *promoting* slippage. In alternative hypotheses, slippage events naturally arising from random DNA metabolic activities are assumed to be stabilized by intercalating agents [e.g., Streisinger and Owen (1985)].

Several lines of evidence suggest that ICR-191 may inflict replication-stopping DNA damage in vivo. For example, ICR-191 induces the *E. coli* SOS functions (Podger & Hall, 1985); ICR-191 is more mutagenic in excision-deficient bacteria (Newton et al., 1972; Imray & MacPhee, 1976), a property associated with bulky SOS-inducing mutagens.

Our observations suggest that ICR-191 may preferentially attack guanines occurring in runs. Similarly, the reactivity of quinacrine mustard (a structural relative of ICR-191 with an identical acridine ring) with some of the guanines within four guanine runs may be as high as 10 times that of guanines that do not occur in runs (Mattes et al., 1986). Such preferential attack probably contributes to frame-shift induction by ICR-191. However, it is unlikely that damage preference alone is sufficient to account for the magnitude of the frame-shift mutagenic effect at the 4 bp guanine runs and the 5 bp run, because the combined damage at the large number of 2 and 3 bp guanine runs (which also are favored over single guanines) as well as at single guanines should exceed that at the hot spots. The fact that virtually no frame shifts are observed at runs of less than four guanines indicates that efficient frame-shift mutagenesis must require a second property of base runs which correlates nonlinearly with the length of the run. This property is most likely to be the propensity of runs to form misaligned structures (Streisinger & Owen, 1985).

Table III: Analysis of the Base Substitution Specificity of Guanine Damage in DNA

type of lesion	G·C > A·T	G·C > T·A	G·C > C·G	(G·C > T·A)/(G·C > A·T)
aguaninic sites[a]	14	27	7	1.93
AFB1-oxide[b]	15	13	4	0.87
AFB1-Cl_2[c]	15	12	0	0.8
AFB1-Cl_2 (FAPY)[d]	11	19	4	1.73
ICR-191	20	10	0	0.5

[a] Data of Kunkel (1984). [b] AFB1-2,3-oxide, the natural activated form; data of Sahasrabudhe et al. (1989). [c] AFB1-2,3-dichloro, an analogue of the activated form of AFB1; data of Sambamurti et al. (1988). [d] Ring-opened form of the adduct (formamidopyrimidine-AFB1-Cl_2); data of Sambamurti et al. (1988).

Figure 5 summarizes some possible mechanisms for frame-shift induction by ICR-191. In these simplified models, replication is shown as stopping opposite the adducted guanine, and the acridine is arbitrarily shown as stacking 5′ of the affected guanine. Support for the asymmetric stacking depicted in structures A.6 and A.7 comes from the work of Sakore et al. (1979) and of Young and Kallenbach (1981). Extensions of the Young and Kallenbach (1981) model on how 9-aminoacridine can be positioned opposite a cytosine in a duplex run of G·C bp support the structures shown in A.6 and A.7. The specificity of ICR-191 for +1 frame shifts at the same hot spot sequences where AFB1 is known to induce both −1 and +1 frameshifts (Refolo et al., 1987; Sambamurti et al., 1988) suggests that the ICR-191 specificity may be lesion-dependent at least for these sites. Two classes of mechanisms can account for this specificity: (a) inhibition of template slippage by the lesion; e.g., the acridine moiety stabilizes the stack of guanines through an interaction such as stacking between two adjacent guanines in the run or by an "outside" interaction which sterically hinders a template slippage event (Figure 5); (b) inefficient recovery of −1 frame shifts; e.g., mismatch repair of +1 frame shifts may be selectively inhibited. Figure 5 (A.6 and A.7) suggests how a misalignment can occur without an extrahelical base, which may help in eluding recognition by the mismatch repair system.

Base Substitutions Induced by ICR-191. Table III presents an analysis of the specificity of the mutations induced by ICR-191, AFB1, and abasic sites in the M13 LacZ(α) gene. Abasic sites are believed to exemplify noninstructional lesions bypassed by the frequent misinsertion of an A. Accordingly, Table III shows that G·C to T·A transversions predominate among mutations induced by abasic sites in the M13 system [data of Kunkel (1984)]. As shown in the last column of Table III, the transversion to transition ratio [(G·C > T·A)/(G·C > A·T)] for abasic sites is 1.9. This ratio for primary AFB1 lesions drops to 0.8–0.9, suggesting that misinsertion of A is not a predominant event opposite AFB1 damage. As previously discussed (introduction), G·T mispairing promoted by guanine N7 adduction through guanine N1 deprotonation can account for this specificity. On the other hand, conversion of the primary AFB1 lesions to the guanine imidazole ring opened

form should abolish N1 deprotonation and, therefore the ability to mispair with T. Consistent with this possibility, the transversion to transition ratio for the ring-opened AFB1 "FAPY" lesions reverts to 1.7, a ratio similar to that for a number of noninstructional lesions. Table III also shows that this ratio is 0.5 for ICR-191, reflecting the preferential bypass of lesions by a misincorporation of T. This specificity, therefore, is consistent with the hypothesis that a mechanism of base substitution mutagenesis by bulky guanine N7 lesions is mispairing with thymine.

Three hypothetical alternative premutagenic lesions that can give rise to the above specificity are (a) a guanine lesion other than the N7 adduct capable of G·T mispairing, (b) a cytosine lesion capable of C·A mispairing, and (c) a noninstructional cytosine lesion bypassed by the "A" rule. Irrespective of which lesion(s) are mutagenic, the data here make it clear that some bulky mutagens can indeed preferentially induce G·C to A·T transitions. While there is evidence that guanine N7 atom is the principal site of reaction for this class of alkylating agents and that ICR-191 treatment specifically induces guanine-targeted alkali-labile sites, adduct characterization and site-specific adduction approaches may be required to confirm that these lesions represent important premutagenic lesions. Even though the possibility that a part or all of the observed substitutions may be caused by DNA lesions other that guanine N7 adducts cannot be overlooked, available information on ICR-191 as well as AFB1 is consistent with the above interpretation. We are presently investigating whether the alternative mechanisms outlined above can account for the observed specificity of base substitution mutagenesis by ICR-191.

Registry No. ICR 191, 17070-45-0.

References

Basu, A. K., & Essigmann, J. M. (1988) *Chem. Res. Toxicol. 1*, 1–18.

Busby, W. F., Jr., & Wogan, G. N. (1984) in *Chemical Carcinogenesis* (Searle, C. E., Ed.) pp 945–1136, American Chemical Society, Washington, DC.

Calos, M. P., & Miller, J. H. (1981) *J. Mol. Biol. 153*, 39–66.

Heilig, J. S. (1989) in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K., Eds.) pp 1.7.1–1.7.11, John Wiley & Sons, New York, NY.

Imray, F. P., & MacPhee, D. G. (1976) *Mutat. Res. 34*, 35–42.

Kunkel, T. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1494–1498).

Kunkel, T. A., & Soni, A. (1988) *J. Biol. Chem. 263*, 14784–14789.

Larson, K., Sahm, J., Shenkar, R., & Strauss, B. (1985) *Mutat. Res. 150*, 77–84.

Lawley, P. D., & Brookes, P. (1961) *Nature 192*, 1081–1082.

LeClerc, J. E., Istock, N. L., Saran, B. R., & Allen, R. (1984) *J. Mol. Biol. 180*, 217–237.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning—A Laboratory Manual*, pp 475–478, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Mattes, W. B., Hartley, J. A., & Kohn, K. W. (1986) *Nucleic Acids Res. 24*, 2971–2987.

McCann, J., Spingarn, J. E., Kobori, J., & Ames, B. N. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 979–983.

Newton, A., Masys, D., Leonardi, E., & Wygal, D. (1972) *Nature, New Biol. 236*, 19–22.

Podger, D. M., & Hall, R. M. (1985) *Mutat. Res. 142*, 87–91.

Rabkin, S. D., & Strauss, B. S. (1984) *J. Mol. Biol. 178*, 569–594.

Refolo, L. M., Bennett, C. B., & Humayun, M. Z. (1987) *J. Mol. Biol. 193*, 609–636.

Sahasrabudhe, S. R., Sambamurti, K., & Humayun, M. Z. (1989) *Mol. Gen. Genet. 217*, 20–25.

Sakore, T. D., Reddy, B. S., & Sobell, H. M. (1979) *J. Mol. Biol.* 135, 763–785.

Sambamurti, K., Callahan, J., Luo, X., Perkins, C. P., Jacobsen, J. S., & Humayun, M. Z. (1988) *Genetics 120*, 863–873.

Skopek, T. R., & Hutchinson, F. (1984) *Mol. Gen. Genet. 195*, 418–423.

Streisinger, G., & Owen, J. (1985) *Genetics 109*, 633–659.

Vieira, J., & Messing, J. (1982) *Gene 19*, 259–268.

Walker, G. W. (1984) *Microbiol. Rev. 48*, 60–93.

Young, P. R., & Kallenbach, N. (1981) *J. Mol. Biol. 145*, 785–813.