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Genetic Toxicology and Environmental Mutagenesis

Comparison of SYBR® Green I nucleic acid gel stain mutagenicity and ethidium bromide mutagenicity in the *Salmonella*/mammalian microsome reverse mutation assay (Ames test)

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Abstract

SYBR® Green I nucleic acid gel stain is an unsymmetrical cyanine dye developed for sensitive detection of nucleic acids in electrophoretic gels. Its mechanism of nucleic acid binding is not known, whereas the most commonly used nucleic acid gel stain, ethidium bromide, is a well-characterized intercalator. We compared the mutagenicity of SYBR Green I stain with that of ethidium bromide in *Salmonella*/mammalian microsome reverse mutation assays (Ames tests). As expected [J. McCann, E. Choi, E. Yamasaki, B.N. Ames, Proc. Natl. Acad. Sci. USA, 72 (1975) 5135–5139], ethidium bromide showed high revertant frequencies in several frameshift indicator strains (averaging 68-fold higher than vehicle controls in TA98, 80-fold higher in TA1538, 15-fold higher in TA1537, and 4.4-fold higher in TA97a), only in the presence of rat liver extracts (S9). Small increases in revertant frequencies were observed for ethidium bromide in the base-substitution indicator strain TA102 both in the presence and absence of S9 (averaging 2.0- and 1.8-fold higher than vehicle controls, respectively) and in base-substitution indicator strain TA100 in the presence of S9 (averaging 1.6-fold higher than vehicle controls). A small mutagenic effect was detected for SYBR Green I stain in frameshift indicator strain TA98 (averaging 2.2-fold higher than vehicle controls) only in the absence of S9 and in base-substitution indicator strain TA102, both in the presence and absence of S9 (averaging 2.2- and 2.7-fold higher than vehicle controls, respectively). Thus, SYBR Green I stain is a weak mutagen and appears to be much less mutagenic than ethidium bromide. These results suggest that SYBR Green I stain may not intercalate, and if it does, that its presence does not give rise to point mutations at a high frequency.

Keywords: SYBR Green I; Mutagenicity; Ethidium bromide; Ames test; Gel stain; Intercalation; Frameshift indicator

1. Introduction

The phenanthridium dye, ethidium bromide, is the stain that is most commonly used for directly detecting nucleic acids in electrophoretic gels. This popularity is due in part to the fact that, although unbound

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ethidium bromide has significant intrinsic fluorescence, the dye displays a 20–25-fold increase in fluorescence upon intercalation into double-stranded (ds) regions of nucleic acids [1]. A novel unsymmetrical cyanine dye, SYBR® Green I nucleic acid gel stain, has been developed recently [2,3], which has several distinct advantages over ethidium bromide. First, it has minimal intrinsic fluorescence and exhibits a 800- to 1000-fold fluorescence enhancement and high quantum yield (~ 0.8) upon binding to ds DNA [2,3]. Second, the affinity of SYBR Green I stain for ds DNA is approximately 100-fold higher affinity than that of ethidium bromide. Its affinity is so high that the dye can be used as an electrophoretic prestain [3] like the structurally related unsymmetrical cyanine dimer dyes, YOYO®-1 and TOTO®-1 [4]. Third, when SYBR Green I stain is used as an electrophoretic poststain, it provides a very sensitive method for detecting ds DNA. It has been used to detect 10–20 pg ds DNA per band in standard electrophoretic minigels [2,3]—a sensitivity approximately 25- to 50-fold greater than can be achieved with ethidium bromide. SYBR Green I stain is 50–100 times more sensitive than ethidium bromide for detecting short, single-stranded (ss) oligonucleotides; 1–2 ng of a synthetic 24-mer has been detected in a polyacrylamide gel poststained with the dye [2]. The sensitivity of SYBR Green I stain has made it an important alternative to several established methodologies: to ethidium bromide staining for detecting reverse transcription polymerase chain reaction (PCR) products on agarose gels [5,6]; to radioactive labels for detecting short tandem repeat sequences using polyacrylamide gels [7] and for detecting DNA damage in pulsed field agarose gels [8]; and to silver stain and radioactive labels for a PCR-based telomerase assay [9,10] and for forensic DNA typing [11,12]. The dye has also been used in place of ethidium bromide to develop sensitive gel-based assays [13] and radial diffusion assays [14] for nuclease activity. In capillary electrophoresis, SYBR Green I stain has been shown to allow detection of as little as 80 fg ds DNA, with a linear range extending over more than three orders of magnitude in DNA concentration [15], and to allow sequence-dependent separation of DNA fragments for mutation detection [16]. Finally, SYBR Green I stain has been used with a rapid-cycle thermocycler to quantitate DNA amplification products during the course of the amplification reaction [17,18].

Ethidium bromide has been shown to inhibit replication in several organisms by interfering with both DNA and RNA synthesis (reviewed in Ref. [19]) and causes frameshift mutations in bacteria [20]. These effects are presumably due to the presence on the genomic DNA of covalently bound metabolic or photoactivated products of the parent dye molecule that may intercalate, causing errors during replication (reviewed by Fukunaga et al. [21]). TOTO-1 and YOYO-3 cyanine dimer dyes have been shown not only to intercalate into ds DNA, but also to have complex binding modes that might involve interactions with sites in the major or minor grooves and/or with the phosphate backbone of DNA molecules [22,23]. These observations raise the possibility that these dyes are mutagenic; although mutagenicity test results have not been reported. Thus, based on the structural similarities between SYBR Green I stain and TOTO-1, YOYO-1, and YOYO-3 dyes, it is of interest to determine the mutagenic potential of SYBR Green I stain. The relative safety of SYBR Green I stain is of particular interest because the dye is commercially available only as a solution in dimethylsulfoxide (DMSO), which is known to facilitate entry of organic compounds into cells and tissues [24].

The purpose of this investigation was to compare the mutagenicity of SYBR Green I stain with ethidium bromide using the *Salmonella*/mammalian microsome reverse mutation assay, commonly known as the Ames test [25,26]. This assay evaluates compounds and their metabolites for their ability to induce mutations at specific sites in the histidine operon of several *Salmonella typhimurium* tester strains. Because the binding mode of the SYBR Green I dye is not well understood, the bacterial mutagenicity assay was performed with a battery of strains containing both C–G and A–T base pairs at the critical mutation site. With these tester strains, mutations resulting from base pair substitutions, frameshifts, and DNA crosslinks can be detected. Mutagenicity assays were performed both in the presence and absence of a rat liver microsome preparation (S9) in order to determine the mutagenic potential of the metabolites of SYBR Green I stain as well as of the dye itself.

2. Materials and methods

2.1. Chemicals

SYBR Green I nucleic acid gel stain (CAS #163795-75-3, purity 96%) and ethidium bromide (CAS #1239-45-8, purity 95%) were both from Molecular Probes, Eugene, OR. Histidine, biotin, ampicillin, dimethylsulfoxide (DMSO, CAS #67-68-5, purity > 99%), 2-aminoanthracene (CAS #613-13-8, purity 97.5%), mitomycin C (CAS #50-07-7), sodium azide (CAS #26628-22-8, purity > 98%), KCl, tetracycline, and glucose 6-phosphate were from Sigma, St. Louis, MO. ICR-191 (CAS #1707-45-0, purity > 95%) was from Polysciences, Warrington, PA. 2-Nitrofluorene (CAS #607-57-08, purity 98%) was from Aldrich, Milwaukee, WI. Tryptophan, $MgCl_2$, Na_2HPO_4, and NaH_2PO_4 were from Fisher Scientific, Fair Lawn, NJ. NADP was from Boehringer Mannheim, Indianapolis, IN. Oxoid Nutrient Broth No. 2 (dry powder) was from Unipath, Basingstoke, England. Liver microsomal enzymes (S9 homogenate, Batch 0501, 44.2 mg of protein/ml), bottom agar plates, and overlay agar were purchased from Molecular Toxicology, Annapolis, MD.

2.2. Mutagenicity assay

The Ames assay was performed using the plate incorporation method of exposure as described by Maron and Ames [26]. *S. typhimurium* tester strains TA98, TA100, TA1535, TA1537, TA1538, TA97a, and TA102, as described in Refs. [25–27], were obtained from Dr. Bruce N. Ames, Department of Biochemistry, University of California, Berkeley, CA, USA. Overnight cultures were grown in Vogel–Bonner salt solution [28] supplemented with 2.5% (w/v) Oxoid Nutrient Broth #2; for tester strain TA102, the broth also contained tetracycline (2 μg/ml) to maintain the pAQ1 plasmid copy number. All cultures were removed from incubation when they reached a density of $1–2 \times 10^9$ cells/ml and were checked for genetic integrity as recommended by Maron and Ames [26]. Bottom agar (25 ml/plate) was Vogel–Bonner minimal medium E [28], supplemented with 1.5% (w/v) agar and 0.2% (w/v) glucose. Overlay agar was prepared with 0.7% agar (w/v) and 0.5% NaCl (w/v) and was supplemented with 10 ml of 0.5 mM histidine/biotin solution per 100 ml agar. S9 homogenate was prepared from male Sprague–Dawley rats that had been injected with Aroclor 1254 at 500 mg/kg, 5 days prior to sacrifice [25]. The components of the S9 mix were 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose 6-phosphate, 4 mM NADP, 100 mM sodium phosphate, pH 7.4, and S9 homogenate at a concentration of 0.1 ml/ml of mix (10%). Assays were performed on open lab benches under ordinary room light.

For both compounds, six doses were tested in both the presence and absence of S9 mix, along with the appropriate vehicle and positive controls. Each dose was plated in triplicate. The doses tested were selected based on the results of a dose rangefinding assay. For the mutagenicity assay, 500 μl of S9 mix (or deionized water when S9 mix was not required), 100 μl of tester strain culture, and 50 μl of compound dose (in DMSO) were added to 2.0 ml of overlay agar, which was mixed and overlaid onto a minimal agar plate. Following incubation at 37 ± 2°C for 48 ± 8 h, revertant colonies were counted. The results of the initial mutagenicity assay were confirmed in an independent experiment.

3. Results

The results of the two independent, triplicate mutagenicity assays of ethidium bromide and SYBR Green I stain, presented as mean revertants per plate with standard deviation, are shown in Tables 1–3. Table 4 summarizes the results, presented as fold-increases in mutagenicity obtained with each nucleic acid stain for each tester strain.

3.1. Frameshift activity

3.1.1. Ethidium bromide

Increases in the number of revertants per plate were observed with +1 frameshift mutation-indicating tester strains TA98 [29,30] (maximum increases of 65.7- and 70.9-fold) and TA1538 (maximum increases of 99.6- and 60.7-fold) in the presence of S9 mix (Table 1). Increases in revertant frequencies were also observed with −1 frameshift mutation-indicating tester strains TA1537 (maximum increases

Table 1
Mutagenicity assay results for (+1) frameshift indicator strains TA98 and TA1538[a]

	Dose (μg)[b]	TA98		TA1538	
		Experiment 1	Experiment 2	Experiment 1	Experiment 2
SYBR Green I stain with S9					
Vehicle control		26 ± 8	19 ± 7	14 ± 1	13 ± 2
	0.100	38 ± 7	20 ± 2	18 ± 2	15 ± 5
	0.333	32 ± 14	23 ± 3	16 ± 5	21 ± 5
	1.00	27 ± 8	32 ± 3	23 ± 6	19 ± 9
	3.33	35 ± 4	30 ± 8	29 ± 5	23 ± 4
	10.0 (T)[c]	14 ± 3	19 ± 9	10 ± 5	3 ± 2
	33.3 (T)	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Positive control[d]		1046 ± 94	762 ± 52	1497 ± 57	1163 ± 95
SYBR Green I stain without S9					
Vehicle control		16 ± 1	11 ± 6	10 ± 2	8 ± 4
	0.0333	17 ± 4	11 ± 4	13 ± 4	8 ± 4
	0.100	21 ± 2	18 ± 1	17 ± 4	14 ± 6
	0.333	24 ± 6	18 ± 7	17 ± 1	14 ± 7
	1.00	37 ± 3	23 ± 3	13 ± 2	13 ± 4
	3.33 (T)	21 ± 7	11 ± 2	1 ± 0	0 ± 0
	10.0 (T)	2 ± 3	0 ± 0	0 ± 0	0 ± 0
Positive control		149 ± 6	128 ± 9	309 ± 18	214 ± 4
Ethidium bromide with S9					
Vehicle control		20 ± 5	14 ± 5	11 ± 2	14 ± 5
	0.05	ND[e]	23 ± 2	ND	20 ± 5
	0.10	ND	73 ± 7	ND	83 ± 11
	0.50	ND	128 ± 7	ND	153 ± 42
	1.00	ND	421 ± 11	ND	481 ± 29
	5.00	ND	992 ± 22	ND	850 ± 55
	10.0	720 ± 43	776 ± 72	735 ± 101	687 ± 136
	25.0	ND	ND	759 ± 63	ND
	50.0	1313 ± 130	ND	1096 ± 83	ND
	100	1299 ± 141	ND	945 ± 274	ND
	250	83 ± 12	ND	307 ± 68	ND
	500	1 ± 1	ND	2 ± 2	ND
Positive control		1199 ± 116	1264 ± 2	1413 ± 70	1262 ± 95
Ethidium bromide without S9					
Vehicle control		20 ± 6	14 ± 1	10 ± 4	10 ± 5
	10.0	21 ± 8	19 ± 1	12 ± 2	12 ± 2
	25.0	18 ± 5	17 ± 6	13 ± 4	12 ± 1
	50.0	27 ± 8	21 ± 4	14 ± 5	12 ± 3
	100	22 ± 4	18 ± 5	11 ± 5	6 ± 2
	250	2 ± 3	4 ± 3	0 ± 0	ND
	500 (T)	0 ± 1	0 ± 1	0 ± 0	0 ± 0
Positive control		167 ± 22	150 ± 4	254 ± 15	287 ± 7

[a] Mean number of revertants per plate ± standard deviation, for experiments performed in triplicate (see Section 2).
[b] Dosage indicates the amount of test material applied per plate.
[c] (T) indicates that toxicity (as indicated by background bacterial lawn thinning compared with vehicle control plates) was observed with this dose of test material under this test condition.
[d] Positive controls for both strains were 2.5 μg/plate 2-aminoanthracene in the presence of S9 extract and 1.0 μg/plate 2-nitrofluorene in the absence of S9 extract.
[e] ND: Not determined.

Table 2
Mutagenicity assay results for (−1) frameshift indicator strains TA1537 and TA97a[a]

	Dose (μg)[b]	TA1537		TA97a	
		Experiment 1	Experiment 2	Experiment 1	Experiment 2
SYBR Green I stain with S9					
Vehicle control		9 ± 2	5 ± 2	156 ± 11	146 ± 17
	0.100	6 ± 3	7 ± 1	154 ± 12	ND[c]
	0.333	10 ± 6	7 ± 3	153 ± 10	149 ± 13
	1.00	10 ± 5	7 ± 2	143 ± 12	139 ± 11
	3.33	8 ± 3	6 ± 1	147 ± 10	107 ± 7
	10.0 (T)[d]	6 ± 1	5 ± 2	114 ± 9	62 ± 1
	33.3 (T)	0 ± 0	0 ± 0	77 ± 12	22 ± 8
	100 (T)	ND	ND	ND	0 ± 0
Positive Control[e]		184 ± 22	189 ± 21	1341 ± 75	1018 ± 32
SYBR Green I stain without S9					
Vehicle control		10 ± 3	5 ± 1	103 ± 4	86 ± 13
	0.0333	10 ± 2	3 ± 2	104 ± 7	ND
	0.100	7 ± 3	7 ± 2	101 ± 3	74 ± 17
	0.333	8 ± 3	6 ± 2	96 ± 11	70 ± 4
	1.00	7 ± 3	6 ± 2	106 ± 11	70 ± 11
	3.33 (T)	4 ± 1	1 ± 1	89 ± 5	45 ± 9
	10.0 (T)	0 ± 0	0 ± 0	57 ± 6	3 ± 2
	33.3 (T)	ND	ND	ND	0 ± 0
Positive control		97 ± 6	75 ± 12	1573 ± 25	1550 ± 59
Ethidium bromide with S9					
Vehicle control		8 ± 3	8 ± 3	165 ± 13	125 ± 7
	10.0	24 ± 3	25 ± 1	291 ± 7	386 ± 55
	25.0	36 ± 7	35 ± 6	338 ± 9	451 ± 69
	50.0	56 ± 10	55 ± 10	438 ± 18	603 ± 160
	100	66 ± 6	62 ± 4	563 ± 32	598 ± 117
	250	120 ± 2	119 ± 12	667 ± 49	2 ± 2
	500	92 ± 44	86 ± 36	45 ± 7	0 ± 1
Positive control		198 ± 3	221 ± 39	1249 ± 67	1056 ± 44
Ethidium bromide without S9					
Vehicle control		7 ± 3	8 ± 4	100 ± 1	86 ± 7
	5.0	ND	ND	103 ± 4	79 ± 9
	10.0	8 ± 3	6 ± 3	100 ± 7	73 ± 9
	25.0	6 ± 2	11 ± 5	116 ± 12	75 ± 11
	50.0	8 ± 3	6 ± 3	129 ± 5	21 ± 13
	100	11 ± 2	10 ± 3	92 ± 13	1 ± 2
	250	6 ± 1	1 ± 2	0 ± 0	0 ± 0
	500 (T)	0 ± 0	0 ± 0	ND	ND
Positive control		106 ± 20	63 ± 3	1759 ± 48	1420 ± 107

[a] Mean number of revertants per plate ± standard deviation, for experiments performed in triplicate (see Section 2).
[b] Dosage indicates the amount of test material applied per plate.
[c] ND: Not determined.
[d] (T) indicates that background lawn toxicity (as indicated by background bacterial lawn thinning compared with vehicle control plates) was observed with this dose of test material under this test condition.
[e] Positive controls for both strains were 2.5 μg/plate 2-aminoanthracene in the presence of S9 extract and 2.0 μg/plate ICR-191 in the absence of S9 extract.

Table 3
Mutagenicity assay results for base-substitution indicator strains TA100, TA1535, and TA102[a]

	Dose (μg)[b]	TA100		TA1535		TA102	
		Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2
SYBR Green I stain with S9							
Vehicle control		101 ± 9	85 ± 9	13 ± 3	9 ± 2	254 ± 29	285 ± 6
	0.100	97 ± 7	86 ± 12	12 ± 2	11 ± 2	249 ± 30	321 ± 28
	0.333	115 ± 3	79 ± 10	12 ± 6	8 ± 3	288 ± 26	378 ± 4
	1.00	106 ± 9	76 ± 3	8 ± 3	10 ± 1	403 ± 11	444 ± 23
	3.33	98 ± 10	74 ± 6	14 ± 3	11 ± 2	494 ± 19	640 ± 19
	10.0 (T)[c]	83 ± 22	57 ± 3	11 ± 3	8 ± 3	514 ± 44	592 ± 30
	33.3 (T)	12 ± 6	3 ± 1	5 ± 3	4 ± 2	451 ± 72	601 ± 7
Positive control[d]		1205 ± 6	943 ± 100	158 ± 12	131 ± 5	1919 ± 34	1945 ± 22
SYBR Green I stain without S9							
Vehicle control		89 ± 15	72 ± 11	8 ± 3	10 ± 5	197 ± 12	223 ± 16
	0.0333	93 ± 12	74 ± 7	15 ± 5	9 ± 3	218 ± 17	239 ± 14
	0.100	91 ± 10	70 ± 12	10 ± 1	9 ± 3	253 ± 12	306 ± 21
	0.333	76 ± 6	59 ± 15	9 ± 1	6 ± 4	336 ± 18	345 ± 32
	1.00	80 ± 12	56 ± 7	13 ± 3	9 ± 4	462 ± 28	459 ± 30
	3.33 (T)	45 ± 7	37 ± 5	7 ± 2	4 ± 5	548 ± 46	577 ± 55
	10.0 (T)	19 ± 7	0 ± 1	2 ± 3	1 ± 1	157 ± 41	419 ± 31
Positive control		692 ± 35	452 ± 21	467 ± 71	396 ± 3	1098 ± 64	1447 ± 24
Ethidium bromide with S9							
Vehicle control		110 ± 22	94 ± 11	7 ± 5	15 ± 3	251 ± 34	290 ± 10
	10.0	136 ± 20	135 ± 7	11 ± 4	12 ± 3	314 ± 9	349 ± 5
	25.0	146 ± 18	152 ± 4	10 ± 1	13 ± 4	334 ± 22	409 ± 40
	50.0	158 ± 9	164 ± 4	11 ± 5	17 ± 1	366 ± 26	419 ± 18
	100	137 ± 17	148 ± 11	8 ± 2	12 ± 4	484 ± 16	588 ± 27
	250	13 ± 11	33 ± 9	7 ± 3	7 ± 2	441 ± 109	572 ± 118
	500	0 ± 0	1 ± 1	2 ± 1	0 ± 1	1 ± 2	9 ± 3
Positive control		1363 ± 28	ND[e]	175 ± 3	180 ± 9	1963 ± 23	2265 ± 50
Ethidium bromide without S9							
Vehicle control		94 ± 5	83 ± 9	11 ± 2	12 ± 3	173 ± 5	219 ± 4
	5.0	ND	ND	ND	ND	ND	233 ± 18
	10.0	91 ± 5	92 ± 13	12 ± 6	13 ± 1	213 ± 11	285 ± 9
	25.0	117 ± 10	100 ± 3	11 ± 3	9 ± 2	257 ± 30	305 ± 18
	50.0	118 ± 19	109 ± 15	7 ± 4	6 ± 4	269 ± 18	366 ± 26
	100	126 ± 5	110 ± 3	11 ± 2	10 ± 1	309 ± 24	340 ± 25
	250	50 ± 5	36 ± 5	8 ± 3	4 ± 1	1 ± 1	3 ± 2
	500 (T)	7 ± 2	1 ± 2	0 ± 1	0 ± 0	0 ± 0	ND
Positive control		674 ± 37	618 ± 27	620 ± 42	450 ± 32	1228 ± 45	1506 ± 24

[a] Mean number of revertants per plate ± standard deviation, for experiments performed in triplicate (see Section 2).
[b] Dosage indicates the amount of test material applied per plate.
[c] (T) indicates that background lawn toxicity (as indicated by background bacterial lawn thinning compared with vehicle control plates) was observed with this dose of test material under this test condition.
[d] Positive controls for TA100 and TA1535 were 2.5 μg/plate 2-aminoanthracene in the presence of S9 extract and 2.0 μg/plate sodium azide in the absence of S9 extract. Positive controls for TA102 were 15.0 μg/plate 2-aminoanthracene in the presence of S9 extract and 1.0 μg/plate mitomycin C in the absence of S9 extract.
[e] ND: Not determined.

Table 4
Summary comparison of SYBR Green I stain and ethidium bromide mutagenic activity[a]

	+1 Frameshift indicator strains[b]				−1 Frameshift indicator strains				Base-substitution indicator strains					
	TA98 (*his*D3052, *uvr*B) (pKM101)		TA1538 (*his*D3052, *uvr*B)		TA1537 (*his*D3076, *uvr*B)		TA97a (*his*D6610, *uvr*B) (pKM101)		TA100 (*his*G46, *uvr*B) (pKM101)		TA1535 (*his*G46, *uvr*B)		TA102 (*his*G428, pAQ1)	
S9 extract[c]	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no	yes	no
SYBR Green I stain														
Experiment 1	–	2.3	–	–	–	–	–	–	–	–	–	–	2.1	2.8
Experiment 2	–	2.1	–	–	–	–	–	–	–	–	–	–	2.2	2.6
Average	–	2.2	–	–	–	–	–	–	–	–	–	–	2.2	2.7
Ethidium bromide														
Experiment 1	65.7	1.3	99.6	–	15.0	–	4.0	–	1.4	–	–	–	1.9	1.8
Experiment 2	70.9	1.5	60.7	–	14.9	–	4.8	–	1.7	–	–	–	2.0	1.7
Average	68	1.4	80	–	15	–	4.4	–	1.6	–	–	–	2.0	1.8

[a] Maximum fold-increase in mean number of revertants per plate in the presence of the indicated nucleic acid stain, compared to the number observed in the presence of the vehicle controls; '–' indicates that no significant increase over vehicle controls was observed.
[b] Tester strain name and relevant genotype (in parentheses).
[c] Experiments were performed in the presence or absence of S9 extract.

of 15.0- and 14.9-fold) and TA97a (maximum increases of 4.0- and 4.8-fold) in the presence of S9 mix (Table 2). No increases were observed with these strains in the absence of S9 mix.

3.1.2. SYBR Green I stain

No increases in revertant frequencies were observed with frameshift mutation-indicating tester strains TA98 (Table 1), TA1537, or TA97a (Table 2) in the presence of S9 mix. A small increase was observed with tester strain TA98 (maximum increases of 2.3- and 2.1-fold) in the absence of S9 mix (Table 1). Small increases in revertant frequencies (maximum increases of 2.1- and 1.8-fold) were observed with tester strain TA1538 in the presence of S9 (Table 1). No increases were observed with tester strains TA1538 (Table 1), TA1537, or TA97a (Table 2) in the absence of S9 mix.

3.2. Base-substitution activity

3.2.1. Ethidium bromide

Small, reproducible, dose-responsive increases in revertant frequencies were observed with base-substitution-indicating tester strains TA102 (maximum increases of 1.9- and 2.0-fold) and TA100 (maximum increases of 1.4- and 1.7-fold) in the presence of S9 mix (Table 3). In the absence of S9 mix, only tester strain TA102 showed reproducible increases in revertant frequencies (maximum increases of 1.8- and 1.7-fold). No increases were observed with tester strain TA100 in the absence of S9 mix or with strain TA1535 in either the presence or absence of S9 mix (Table 3).

3.2.2. SYBR Green I stain

Small increases in revertant frequencies were observed with tester strain TA102 in both the presence (maximum increases of 2.3- and 2.1-fold) and the absence (maximum increases of 2.8- and 2.6-fold) of S9 mix (Table 3). No increases in revertant frequencies were observed with tester strains TA100 or TA1535 in either the presence or absence of S9 mix (Table 3).

4. Discussion

Both SYBR Green I stain and ethidium bromide bind to nucleic acids and exhibit fluorescence en-

hancements upon doing so. However, the dyes are structurally very different. Ethidium bromide is a phenanthridium dye, and SYBR Green I stain is an unsymmetrical cyanine dye [31]. In addition, ethidium bromide is well known to intercalate into ds DNA, yielding frameshift mutations in Ames tests [20], whereas the binding mode of SYBR Green I stain is unknown. In comparing the behavior of these two compounds in the Ames test, we observed differences in: (1) the subset of tester strains that were sensitive to each compound, (2) the magnitude of the mutagenic responses, (3) the doses at which cytotoxic and mutagenic responses were observed, (4) the effect of S9 extracts, and (5) the effect of the mutagenesis-enhancing pKM101 plasmid. These differences are discussed below.

4.1. Relative mutagenicity of SYBR Green I stain and ethidium bromide

Ethidium bromide induced mutagenic responses in all +1 frameshift mutation-indicating tester strains (TA98 and TA1538) and −1 frameshift mutation-indicating tester strains (TA1537 and TA97a) examined. However, similar to earlier reports [20], we found that this mutagenicity was exhibited only in the presence of S9 mix. SYBR Green I stain did not induce mutagenic responses in −1 frameshift mutation-indicating strains, and showed a weak mutagenic response in strain TA98 only in the absence of S9 mix. This response is likely to be due to the pKM101 plasmid in TA98, because SYBR Green I stain did not show a dose-dependent mutagenic response in strain TA1538, which lacks pKM101 but is otherwise isogenic to TA98 [32]. The plasmid is thought to increase sensitivity of several of the tester strains to mutagens by the error prone, or SOS, DNA-repair system induced by DNA damage [33]. In contrast to SYBR Green I stain, ethidium bromide did not induce higher overall revertant frequencies in strain TA98 than it did in strain TA1538, suggesting that the two dyes induce mutations by different mechanisms.

Neither SYBR Green I stain nor ethidium bromide showed strong mutagenic activity in TA1535 or TA100 (which are isogenic except that TA100 contains the pKM101 plasmid; [23]), although ethidium bromide exhibited a weak mutagenic activity in strain TA100, in the presence of S9 (again probably due to the presence of the plasmid). SYBR Green I stain and ethidium bromide both showed weak, dose-dependent responses to the base-substitution indicator strain TA102. This result is interesting both because this strain contains an A–T base pair at the site of the mutation whereas the other strains all contain G–C base pairs, and because the *his*G428 mutation in this strain is present on a plasmid (pAQ1), so that about 30 copies of the target sequence per cell are available for reversion. In addition, this mutation can be reverted by second-site generation of an ochre suppressor [34]. It is possible that the low levels of mutagenesis detected in strain TA102 in this study were due to the latter phenomenon, although true primary-site reversion is not ruled out. Strain TA102 is also unique among those tested in that it contains the wild type *uvr*B gene and thus has an intact DNA excision repair system, allowing it to detect crosslinking agents [35–37]. It is therefore possible that some mutagenesis induced by ethidium bromide or by SYBR Green I stain detected by TA102 (Table 3) is related to crosslinking. Both agents are photosensitive, and, since these experiments were performed without light-protection, either the dyes or their metabolic products could induce DNA damage at some frequency by forming crosslinks of dye/DNA photoadducts.

The magnitudes of the peak mutagenic responses exhibited by the two dyes are very different. The largest increase in mutagenicity observed with SYBR Green I stain was 2.8-fold, whereas ethidium bromide exhibited up to 99.6-fold maximal increases in mutagenicity. Whether one applies the '2-fold rule' [38] or other means [39,40] of assessing mutagenesis in the Ames assay, our data show unambiguously that ethidium bromide appears to be a relatively strong mutagen and SYBR Green I stain a weak mutagen, when compared with previously studied compounds [20,41].

In the bacterial strains tested, SYBR Green I stain shows higher cytotoxicity per unit mass than does ethidium bromide. The maximal dose of SYBR Green I stain that showed no toxicity was 33.3 μg per plate (toxicity was observed at much lower doses for some strains), whereas toxicity was only observed at doses of 250–500 μg per plate for ethidium bromide. This

higher toxicity is probably due to the fact that SYBR Green I stain readily penetrates living bacteria (and mammalian cells; unpublished results, Molecular Probes), whereas ethidium bromide is efficiently excluded from live cells. Due to its live cell permeability, SYBR Green I stain may also have a lower threshold effective dose for mutagenesis than it would if the dye were impermeant.

The lack of mutagenic activity of SYBR Green I stain in strain TA98 in the presence of S9 enzymes suggests that the relatively weak mutagenic activity observed in the absence of S9 enzymes is eliminated by its conversion to an inactive compound by S9-dependent phase 1 metabolism. The mutagenic activity may arise from the dye itself. In contrast, the fact that the high mutagenic activity of ethidium bromide in strain TA98 required S9 enzymes indicates that metabolites of ethidium bromide entering the cell are the actual mutagens. The actual dose of ethidium bromide metabolic products received by cells in this type of assay, on a per plate basis, is unknown. This dose could in fact be comparable to the doses of SYBR Green I stain found to exhibit mutagenic responses.

4.2. Implications for understanding the binding mode of SYBR Green I stain

The binding mode of SYBR Green I stain to DNA is not known; however, it binds to ds DNA, RNA, and ss deoxyoligonucleotides with high fluorescence quantum yields ([3], unpublished results, Molecular Probes). Ethidium bromide also binds to RNA and oligonucleotides as well as to ds DNA, despite being an intercalator. Presumably such binding is due to a combination of binding modes, including charge interactions between the cationic dye and negatively charged nucleic acid and perhaps intercalation into ds regions of the RNA. Other polycationic dyes, such as TOTO-1 and ethidium homodimer-1, which are capable of bis-intercalation, nonetheless interact with both ds DNA and ss DNA with similar high affinity [42]. Binding to nucleic acids is sensitive to the presence of mono- and divalent cations for both ethidium bromide [1] and SYBR Green I stain [2], presumably because of the cationic nature of both dyes. However, SYBR Green I stain's affinity for nucleic acids is much greater than that of ethidium bromide, allowing it to remain bound to ds DNA during electrophoresis [3]. Despite this high affinity, SYBR Green I stain does not interfere with restriction endonuclease activity when the dye is used at normal gel staining concentrations [3,43]. This observation suggests that the bound dye might not interfere with binding of sequence-specific DNA binding factors, such as those involved in cellular transcription or replication. SYBR Green I stain is also displaced from ds DNA by treatment with sodium dodecyl sulfate and by ethanol precipitation, but not by phenol-, butanol-, or chloroform-extraction (unpublished results, Molecular Probes). Ethidium bromide is efficiently displaced by phenol-, butanol-, and chloroform extraction, but not by treatment with sodium dodecyl sulfate or by ethanol precipitation. This information, taken together with the lack of mutagenic activity observed in tester strains TA1537 and TA97a, suggests that the binding mode of SYBR Green I stain is distinct from that of ethidium bromide. The primary binding mode of SYBR Green I stain may not be intercalation into ds DNA, but may involve surface or groove interactions.

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