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Distribution of the DNA Adducts of 2-Amino-3-methylimidazo[4,5-*f*]quinoline and 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine in the *supF* Gene as Determined by Polymerase Arrest Assay

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The distribution of the adducts of the cooked meat–derived heterocyclic amines 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) was examined in the *supF* gene of PSP189 by a polymerase-arrest assay using thermal-cycle sequencing. The reactive *N*-acetoxy metabolites of both compounds showed an overwhelming preference for reacting with guanine residues in the *supF* gene of the shuttle vector pSP189. The distribution of the IQ and PhIP guanine adducts was not random; instead, patterns of adduct hot-spots and cold-spots were observed. There was a striking similarity between both compounds in their preferred sites of adduct formation. The finding that IQ and PhIP adducted to guanine concurred with previous results showing that the target sites for IQ and PhIP mutations in *supF* were also at guanine. However, the adduct hot-spot sites were not predictive of the known sites of mutation hot-spots. In addition, despite the similarity in adduct hot-spots for IQ and PhIP, their reported mutation spectra in the *supF* gene were different. Factors in addition to adduct location therefore appear to play a role in the mutation spectra induced by the heterocyclic amines in the *supF* gene.

Key words: Thermal-cycle sequencing, mutagenesis, heterocyclic amines, ^{32}P postlabeling, DNA polymerase

INTRODUCTION

The heterocyclic amines 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (Figure 1) are mutagens and carcinogens found in cooked meats such as beef and fish [1–6]. These compounds undergo metabolic activation via *N*-hydroxylation and esterification to reactive *N*-acetoxy metabolites that covalently modify DNA [7–10]. The formation of DNA adducts has been implicated in the mutagenicity and carcinogenicity of these compounds [1,2,7]. Both compounds have been shown to form a major DNA adduct at the C8 position of guanine [10–14]. A minor DNA adduct of IQ has also been identified at the N^2 atom of guanine [12,13].

Recently we examined the mutagenic specificity of PhIP and IQ in the *supF* gene [15] using the shuttle vector system developed by Seidman et al. [16]. The overwhelming majority of the mutagenic lesions induced by PhIP and IQ were single-base substitutions at guanine. With both compounds, the predominant mutation found at guanine was a G:C → T:A transversion. Despite the similarities among PhIP and IQ in the types of mutations produced, however, the mutation spectra induced by these carcinogens were distinct.

In the study reported here, we used a polymerase-arrest assay with thermal-cycle sequencing to compare the sequence specificity of adduct formation of the reactive metabolites *N*-acetoxy-IQ and *N*-acetoxy-PhIP in the *supF* gene. This study was undertaken to apply a method to detect heterocyclic amine adducts in specific genes, to learn more about the distribution of IQ and PhIP adducts in a DNA sequence, and to gain insight into whether variations in PhIP and IQ adduct distribution in the *supF* gene might contribute to the differences in the mutation spectra observed with these compounds.

MATERIALS AND METHODS

Chemicals, Plasmid, and Primers

N-Hydroxy-IQ and *N*-hydroxy-PhIP were synthesized by reduction of the respective nitro derivatives as described previously [14,17]. The *supF* shuttle vector pSP189 [18] and the primer for the transcribed strand of the *supF* gene [19] were generously provided by Drs. C. N. Parris (National Cancer Institute, Bethesda, MD) and M. M. Seidman (Otsuka Pharmaceutical Co., Rockville, MD). Another primer for the nontranscribed strand of the *supF* gene (the 21-*mer* 5′-GGGCGGAGCCTATGGAAAAAC-3′) was synthesized, purified, and provided by Operon Technologies, Inc (Alameda, CA).

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Abbreviations: IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine.

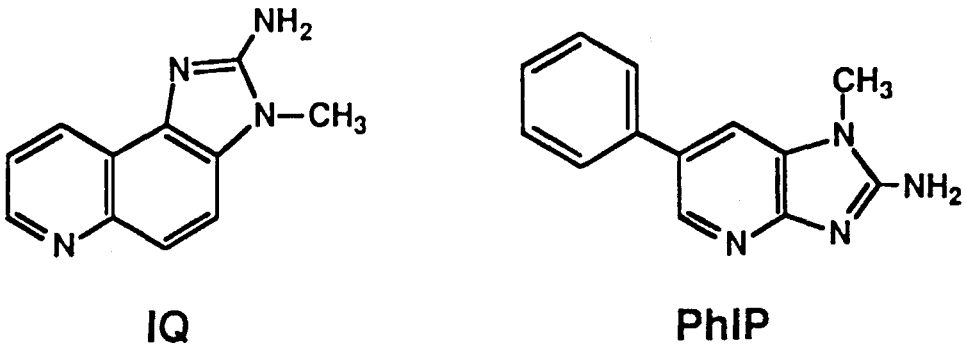

Figure 1. Chemical structures of IQ and PhIP.

Treatment of pSP189 with Carcinogens

Cesium chloride–purified pSP189 (20 μg) was treated in vitro with 5, 10, and 40 μM *N*-hydroxy-IQ or *N*-hydroxy-PhIP in the presence of a fivefold molar excess of acetic anhydride (in 0.7 μL of ethanol) in a total volume of 100 μL of TE buffer (10 mM Tris and 1 mM EDTA, pH 8) as described previously [15]. The acetic anhydride was present to generate the reactive *N*-acetoxy ester derivatives in situ. The incubation was performed at room temperature for 30 min. Solvent control DNA was treated with 200 μM acetic anhydride without carcinogen. After incubation, plasmid DNA was purified from unbound carcinogen by a standard DNA phenol–chloroform–isoamyl alcohol extraction procedure followed by ethanol precipitation and washing with 70% ethanol [20]. Treated plasmid, solvent control plasmid, and untreated plasmid were then used in the polymerase-arrest assay with thermal-cycle sequencing to determine the sites of carcinogen adduction. ^{32}P-Postlabeling analysis of plasmid DNA was also performed to measure total adduct levels.

Polymerase-Arrest Assay with Thermal-Cycle Sequencing

The primers were end-labeled with T4 polynucleotide kinase (Epicentre Technologies, Madison, WI) and [γ-^{32}P]ATP (3000 Ci/mmol) (DuPont NEN, Boston, MA) according to the manufacturer's instructions.

The arrest assay was run with SequiTherm thermostable DNA polymerase and a SequiTherm Cycle Sequencing Kit (Epicentre Technologies). The DNA sequencing protocol provided by the manufacturer was followed, with two modifications. First, the arrest assay was run in the absence of the termination mix of dideoxynucleotides. Instead, 15 μM each dNTP (dATP, dGTP, dCTP, and dTTP) was added to the polymerase reaction mixture. Second, the concentration of $MgCl_2$ in the polymerase reaction mixture was reduced from 2.5 mM to 0.25 mM to reduce background arrest sites. During thermal cycling, the amount of each template DNA (control pSP189 and adducted pSP189) in each reaction was 10 ng. The polymerase reaction was performed for 30 cycles of 30 s denaturation at 95°C, 30 s annealing at 50°C, and 1 min synthesis at 70°C. For sequencing, the amplified template DNA was electrophoresed on a 6% polyacrylamide/7 M urea gel. Concomitantly, cycle sequencing was also performed on unadducted template pSP189 with the dideoxy G, A, T, and C termination mix.

After electrophoresis, the gels were dried, and the intensity of each band was measured with a PhosphoImager (model 425E) equipped with ImageQuant software (v. 3.3) (Molecular Dynamics, Inc., Sunnyvale, CA).

^{32}P-Postlabeling Analysis

The levels of IQ- and PhIP-DNA adducts in the plasmids were determined by ^{32}P-postlabeling analysis essentially as described by Gupta et al. [21]. This method resolves [^{32}P]ATP-labeled phosphonucleotide adducts of IQ and PhIP as fingerprints on autoradiograms after chromatography on polyethyleneimine cellulose thin-layer sheets. ^{32}P-Postlabeling analysis was performed under standard labeling conditions by using the previously described chromatography conditions for IQ- and PhIP-DNA adducts [22–24]. The profiles of the IQ- and PhIP-DNA adducts observed in pSP189 were identical to those reported previously [15].

Stability of IQ and PhIP Adducts During Thermal Cycling

To determine whether IQ- and PhIP-DNA adducts were stable under the conditions of thermal cycling, a sample of pSP189 containing IQ or PhIP adducts was analyzed by ^{32}P-postlabeling before and after thermal cycling. The conditions for thermal cycling were the same as described above except that DNA polymerase and primer were omitted.

RESULTS

Figure 2 shows autoradiograms from representative sequencing gels showing sites of arrest of SequiTherm DNA polymerase during primer extension of either strand of the *supF* gene. The primer was extended until the polymerase encountered an IQ or PhIP adduct. The resultant ^{32}P-labeled transcripts were separated by electrophoresis. Dideoxy sequencing products of the *supF* gene were

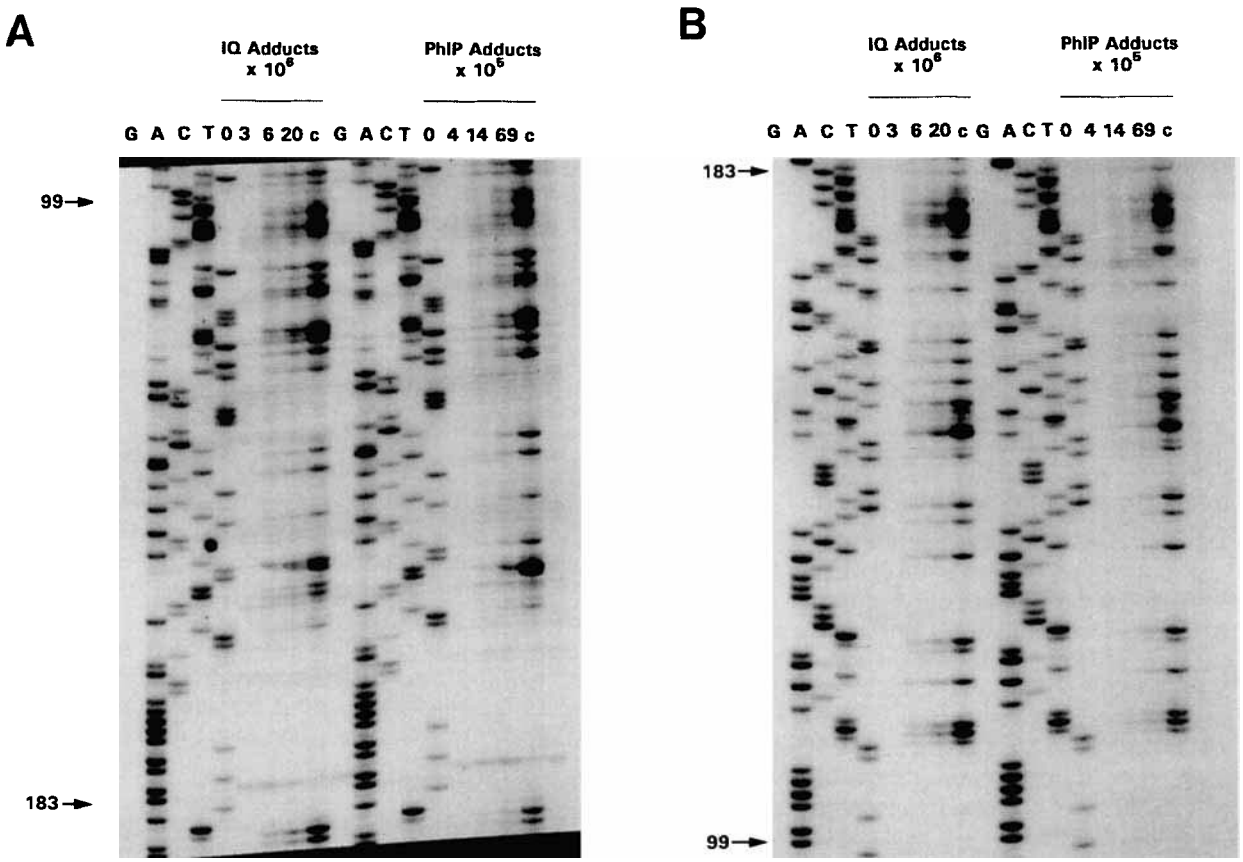

Figure 2. Autoradiographs of DNA sequencing gels showing the sites of polymerase arrests induced by IQ- and PhIP-DNA adducts in the transcribed strand (A) and the nontranscribed strand (B) of the *supF* gene. The DNA sequence of the *supF* gene is shown in both panels, and nucleotide numbers are indicated with arrows on the left side of each panel. The corresponding nucleotide sequence of the *supF* gene is shown in Figure 3. The IQ- and PhIP-DNA adduct levels in the *supF* plasmid (pSP189) were determined by ^{32}P-postlabeling analysis and are reported above the lanes as adducts per 10^5 or 10^6 nucleotides. These values are the total adduct levels observed after treating the plasmids with 5, 10, or 40 μM the respective *N*-hydroxylamine. Lane c, untreated control *supF* (pSP189) DNA; lane 0, *supF* (pSP189) treated with acetic anhydride in the absence of carcinogen (i.e., vehicle control). The letters G, A, C, and T correspond to the dideoxy sequencing termination mix used to determine the *supF* DNA sequence.

also electrophoresed on the same gels to aid in determining the sites of polymerase arrest in the *supF* gene.

As shown in Figure 2, neither the solvent control nor control pSP189 template caused premature polymerase arrest. In contrast, when pSP189 was exposed to either *N*-acetoxy-PhIP or *N*-acetoxy-IQ, there were many polymerase arrest sites, as evidenced by the presence of bands on the sequencing gel. With both IQ and PhIP, the intensity of the bands increased as the adduct level in the template (as determined by ^{32}P-postlabeling analysis of adducted pSP189) increased. The absence of arrest sites in the control DNA and the increase in band intensity observed with increasing adduct levels support the conclusion that the polymerase was blocked by the presence of heterocyclic amine adducts at sites on the template DNA. ^{32}P-Postlabeling of adducted pSP189 before and after thermal cycling showed that there were no qualitative or quantitative changes in IQ or PhIP adducts. These ^{32}P-postlabeling findings support the conclusion that the adducts were stable during thermal cycling. As reported previously [15,24], both IQ- and PhIP-DNA adducts were resolved as three-guanine adduct spots, and the C8-guanine adduct appeared to be the major adduct for both compounds.

Comparison of the arrest sites found in the adducted templates with those seen in the dideoxy sequencing lanes showed that there was a resemblance between the polymerase arrest caused by the heterocyclic amine adducts and that caused by ddCTP incorporation. However, the bands produced by the adducted templates were one nucleotide shorter than those observed by ddCTP termination. This finding supports that the IQ and PhIP adducts were formed at the guanine base and that the polymerase was terminated one base prior to the guanine adduct site. ^{32}P-Postlabeling analysis of pSP189 reacted with *N*-acetoxy-IQ and *N*-acetoxy-PhIP further supported the notion that DNA adducts of IQ and PhIP are formed only on the guanine base (see also refs. 15 and 24). With both IQ and PhIP, the arrest sites were similar at all adduct concentrations examined. Thus, it appears that quantitative changes at guanine adduct sites, rather than qualitative

changes in adduct distribution, occurred at different adduct levels.

We compared the IQ- and PhIP-DNA adduct arrest sites found in the transcribed and nontranscribed strands of the *supF* gene (Figure 3). There was a striking similarity between the polymerase-arrest sites caused by adducts of IQ and PhIP, suggesting that the two compounds showed similar preferences for sites of DNA adduct formation. In addition to the similarity in arrest sites, the magnitudes of the relative intensities at the various arrest sites were similar for the two compounds. Sites of strong (or weak) arrest for IQ were also sites of strong (or weak) arrest for PhIP.

The overwhelming majority of IQ- or PhIP-induced polymerase-arrest sites, as measured by relative band intensity, corresponded to guanine bases in the *supF* gene (Figure 3). The average relative band intensity of arrest sites corresponding to IQ and PhIP adducts at guanine was between 1.6 and 2. Many guanine bases in the *supF* gene showed some degree of IQ or PhIP adduct formation; however, differences in the relative intensities of IQ or PhIP arrest sites were observed among different guanine bases. There were four guanine bases (sites 172, 173, 181, and 182) at which polymerase arrest was not detected for either IQ or PhIP. IQ and PhIP adduct hot-spots (defined as sites where the band intensity in the arrest assay was approximately twofold higher than the average guanine band intensity) were observed at sites 109, 110, 118, 122, 142, 144, and 146. There was one cytosine (site 144) at which an apparent arrest band was seen for IQ and PhIP. However, this base was adjacent to a strong guanine arrest site and may be related to the resolution of the band corresponding to the guanine site. Notably, arrest sites were absent at other cytosines and absent in pyrimidine-rich sequences, such as at sites 167–182. Also, IQ and PhIP adduct arrest sites were not detectable at adenine or thymine. Thus, the N-acetoxy esters of IQ and PhIP did not appear to form adducts at pyrimidines or adenine in the *supF* gene.

The previously determined mutation hot-spots for IQ and PhIP in repair-deficient human fibroblasts (XP12BE(SV40)) or repair-proficient human fibroblasts (GM0637) [15] are represented as boxes and circles, respectively, underneath the DNA sequences in Figure 3. Ninety-seven percent of all single-base mutations and all of the mutation hot-spots were located at guanines [15]. Of 11 total mutation hot-spots for IQ and PhIP in XP12BE(SV40) and GM0637 cells, all except one hot-spot corresponded to a guanine site that showed detectable adduct levels. The one exception was an IQ mutation hot-spot at guanine site 182 that did not produce an arrest band of significant intensity (Figures 2 and 3). The reason for this exception is not known. It is tempting to speculate that the endogenous cellular DNA polymerase was also not arrested at this adduct site and that it consequently placed an incorrect base across from the adduct site, resulting in a mutation. As summarized in Table 1, mutation hot-spots did not necessarily correspond to adduct hot-spots. Whereas the mutational hot-spot for PhIP at site 129 showed an intensity slightly above average, mutation hot-spots at 139 and 179 showed lower-than-average arrest-site band intensities. In the case of IQ, three of eight mutation hot-spots showed slightly higher than average band intensities, while the other five showed lower than average band intensities. Furthermore, the IQ and/or PhIP adduct hot-spots at 109, 110, 118, 122, 142, 144, and 146 did not correspond to mutational hot-spots for either compound.

DISCUSSION

DNA polymerase–arrest assays have been used to determine the location of many chemical carcinogen adducts in DNA sequences [25–30]. Here, we examined the distribution of adducts of the heterocyclic amine food mutagens IQ and PhIP in the *supF* gene using a polymerase-arrest assay with thermal-cycle sequencing. To our knowledge, there have been no previously reported studies of the distribution of IQ- and PhIP-DNA adducts in a DNA sequence by thermal-cycle sequencing. The major advantages of the thermal-cycle sequencing method over conventional polymerase-arrest assays are the substantially lower amounts of DNA needed and the precision and simplicity provided by the thermal cycler. Using this method, we showed that IQ- and PhIP-DNA adducts are detectable in a gene sequence as bands on a sequencing gel. As has been noted previously in polymerase-arrest assays with other carcinogen adducts [25–30], the intensity of the bands appears to reflect the degree of adduct formation at a specific site. This conclusion is further supported by our results showing a relative absence of arrest sites in control DNA and an increase in band intensities with increasing adduct levels (Figure 2).

Using the ^{32}P-postlabeling assay or high-performance liquid chromatography analysis, several studies have demonstrated that the reactive metabolites of IQ and PhIP form adducts at guanine [10–14]. These techniques, however, are not generally applicable to analyzing the distribution of heterocyclic amine adducts in a specific gene. Previously, our laboratory reported the use of a Southern hybridization method to detect heterocyclic amine adducts in specific genes [31], but this method could not distinguish between type or location of heterocyclic amine adducts in the gene. With the polymerase-arrest assay described here, we were able to determine that the N-acetoxy esters of IQ and PhIP formed adducts on the guanine base and that the frequency of guanine adduct formation was not distributed randomly throughout the *supF* gene. Indeed, there were several preferred guanine adduct hot-spot sites for IQ and PhIP: sites 109, 110, 118, 122, 142, 144, and 146. Since adduct hot-spot sites were seen at guanines adjacent to any of the four bases, there did not appear to be a strongly preferred base sequence context for these guanine adduct hot-spots. Five of the seven adduct hot-spots, however, were at GG sites. It is notable that at increasing levels of IQ or PhIP adducts, there were quantitative increases in band intensities rather than qualitative changes in band sites (Figure 2). These findings suggest that the preferred sites of IQ and PhIP

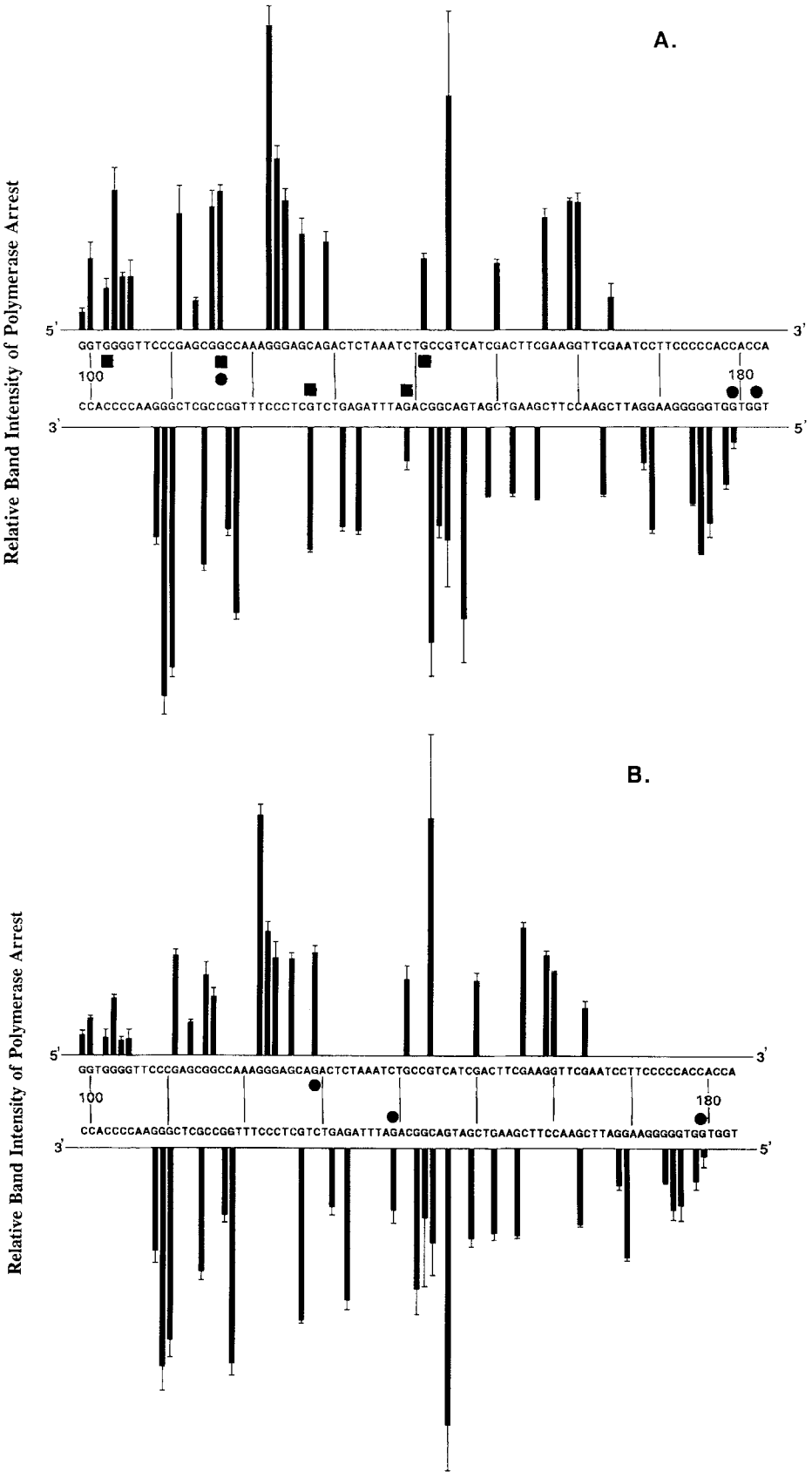
A.
Relative Band Intensity of Polymerase Arrest
5'
3'
GGTGGGGTTCCCGAGCGGCCAAAGGGAGCAGACTCTAAATCTGCCGTCATCGACTTCGAAGGTTCGAATCCTTCCCCCACCACCA
100
180
CCACCCCAAGGGCTCGCCGGTTTCCCTCGTCTGAGATTTAGACGGCAGTAGCTGAAGCTTCCAAGCTTAGGAAGGGGGTGGTGGT
3'
5'
B.
Relative Band Intensity of Polymerase Arrest
5'
3'
GGTGGGGTTCCCGAGCGGCCAAAGGGAGCAGACTCTAAATCTGCCGTCATCGACTTCGAAGGTTCGAATCCTTCCCCCACCACCA
100
180
CCACCCCAAGGGCTCGCCGGTTTCCCTCGTCTGAGATTTAGACGGCAGTAGCTGAAGCTTCCAAGCTTAGGAAGGGGGTGGTGGT
3'
5'

Figure 3.

Table 1. Intensity of Polymerase Arrest at IQ and PhIP Mutational Hot-Spots in the *supF* Gene*

Compound	Mutation hot-spot	Cell type†	Relative intensity/ average relative intensity‡
IQ	102	XP	0.4
	116	XP	1.3
	116	GM	1.3
	127	XP	1.5
	139	XP	0.4
	141	XP	0.7
	179	GM	0.2
	182	GM	–
PhIP	129	GM	1.2
	139	GM	0.7
	179	GM	0.01

*Mutational hot-spot data are from Endo et al. [15].
†XP, XP12BE(SV40); GM, GM0637.
‡The values are expressed as the ratio of the relative intensity observed at the mutation hot-spot to the average relative intensity observed at guanine. The average relative intensitites ± the standard error of the mean (n=3) for guanine in the nontranscribed and transcribed strands were 1.79 ± 0.09 and 1.59 ± 0.02, respectively, for IQ and 1.98 ± 0.05 and 1.61 ± 0.04, respectively, for PhIP.

adduct formation are similar at low and high carcinogen-adduct levels. In addition, since the intensity of the band arrest sites were nearly identical for the adducts of IQ and PhIP, the results show that the distributions of IQ and PhIP adducts in the *supF* gene are strikingly similar (Figure 3, compare panels A and B).

Previously, we characterized the sites of mutations of IQ- and PhIP-DNA adducts in the *supF* shuttle vector system [15]. We observed that 97% of mutations, which included primarily single-base changes such as G → T transversions, occurred at guanine. The findings reported here in conjunction with previous mutation studies [15] support the idea that guanine adducts are responsible for the mutations induced at guanine in the *supF* gene. For example, adduct hot-spot sites 109, 110, 118, 122, 142, 144, and 146 did, in general, correspond to mutation sites for IQ and/or PhIP in either XP12BE(SV40) or GM0637 cells. In addition, guanine arrest at sites 112–115 concurred with mutations at these guanine bases. Thus, mutation sites along the *supF* gene appeared to correlate with adduct sites. However, there appeared to be no correlation between the intensity at an arrest site, i.e., the adduct level at a site, and a mutation hot-spot (Table 1). This has also been previously observed with other carcinogens such as aflatoxin B_1 and benzo[c]phenanthrene [27,28]. Thus, as is the case with other carcinogens, the sites of high IQ and PhIP adduct levels are not the sites of frequent mutations. An implication of this finding is that total adduct levels in a gene may not be directly related to mutagenesis at specific sites within that gene. Mutations at hot-spots might be induced by relatively low adduct levels, and alternately, high adduct levels might be sustained at certain sites without causing a high rate of mutation.

Despite the similarity in adduct distribution between IQ and PhIP, the mutation spectra induced by these compounds were distinct. For example, the guanine at site 127 was apparently an adduct hot-spot for either IQ or PhIP but was a mutation hot-spot only for IQ (in XP12BE(SV40) cells). Our assay, however, did not distinguish between different possible guanine adducts of IQ and PhIP, for example, the N^2-guanine and C8-guanine adducts of IQ. The role that distinct guanine adducts play in the mutagenic spectra of IQ and PhIP deserves further study.

It is generally recognized that mutagenesis does not occur randomly. In the *supF* shuttle vector system and other genes, patterns of hot-spots and cold-spots have been observed with many mutagens [15,16,25–29,32,33]. As the distribution of IQ and PhIP adducts in the *supF* gene is similar despite the differences in their mutation spectra, it appears that other factors in addition to the location of the adducts play a role in the spectra of mutations induced. Recent studies have supported the role of adduct conformation and sequence context in mutagenesis with chemical carcinogen– and ultraviolet light–induced DNA adducts [19,33–36]. How these factors might influence the differences in mutation spectra observed for IQ and PhIP deserves further study.

Figure 3. Relative intensities of polymerase-arrest sites corresponding to IQ (A) and PhIP (B) adducted nucleotides in the nontranscribed and transcribed strands of the *supF* gene. The DNA sequence of the entire *supF* gene is given. The band intensities observed in the arrest assays (see Figure 2) were quantitated. The relative values given are the means ± the standard errors of three experiments. In the results shown here, the IQ and PhIP adduct levels in the *supF* plasmid (pSP189) were 6 and 7 adducts/10^6 nucleotides, respectively, as determined by ^{32}P-postlabeling analysis. Mutation hot-spots in the *supF* gene, determined by us previously [15], are represented underneath the sequence by circles and boxes: ●, mutation hot-spots in repair-proficient GM0637 cells; ■, mutation hot-spots in repair-deficient XP12BE(SV40) cells. (No mutation hot-spots for PhIP were found in XP12BE(SV40) cells.)

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