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MOLECULAR ANALYSIS OF MUTAGENESIS IN *E. COLI*

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Summary. - *In this paper we present a general strategy that is suitable for the analysis of the forward mutation spectrum induced by any physical or chemical mutagen or carcinogen. The assay is based upon the inactivation of the tetracycline resistance gene located on plasmid pBR322. Plasmid DNA is treated* in vitro *with the mutagen and transformed into the host bacteria of choice. Mutant clones are selected and analysed by sequencing. We present also two techniques that allow the determination of the DNA modification spectrum. The comparison for a given mutagen of the modification spectrum and the induced mutation spectrum permits the identification of hot spot sequences. Using different chemical mutagens (derivatives of the carcinogenic aromatic amide N-2-acetylaminofluorene, cis-platinum, etc.) this assay was found to be able to detect the different classes of mutagenic events: base substitutions, frameshifts, insertions and deletions. The advantages and limitations of this assay are discussed.*

Riassunto (Analisi molecolare della mutagenesi in *E. coli*). - *In questo articolo descriviamo una possibile strategia adatta all'analisi dello spettro di mutazioni in avanti indotte da agenti fisici e chimici mutageni o cancerogeni. Il saggio è basato sull'inattivazione del gene per la resistenza alla tetraciclina presente sul plasmide pBR322. Il DNA del plasmide è trattato* in vitro *con il mutageno e trasformato nel batterio ospite. I cloni mutanti sono selezionati e la loro sequenza analizzata. Presentiamo inoltre due tecniche che permettono la determinazione dello spettro di modificazioni sul DNA. Il paragone per un dato mutageno dello spettro di modificazioni sul DNA e dello spettro di mutazione permette l'identificazione di sequenze "hot-spot". Usando differenti mutageni chimici (derivati dell'amide aromatica cancerogena N-2-acetilaminofluorene, cis-platino, ecc.) abbiamo verificato l'idoneità di questo saggio per l'identificazione di differenti classi di eventi mutageni: sostituzioni di base, "frameshift", inserzioni e delezioni. I vantaggi e i limiti di questo saggio sono discussi.*

Introduction

The conversion of DNA lesions into mutations is an active biochemical process. Due to the remarquable efficiency of the error free repair mechanisms only a very small number of DNA lesions will eventually be processed into a mutation. Under normal conditions, less than one percent of DNA lesions give rise to mutations. This makes the biochemical study of the mechanisms involved in mutagenesis very difficult. As a first molecular approach, the study of the mutational specificity of a given mutagen will provide important informations concerning the mechanisms that are involved. This is particularly true if the analysis of the mutational specificity can be performed in hosts having altered genotypes for repair and (or) mutagenesis. Bacteria are in this respect the organisms of choice, due to the large number of existing repair, replication and recombination mutants.

If one wants to study mutational specificity, an important aspect is to develop assays that have as little as possible biases in the selection procedure of the induced mutants. In fact, mutant selection usually relies on an easily selectable phenotype. Although, the ideal situation would be to analyse mutants without having to rely on a given phenotype, it is technically difficult to achieve. We have developed a forward mutation assay based on the inactivation of the tetracycline resistance gene located on plasmid pBR322 [1]. The inactivation of the tetracycline resistance gene was found to respond to the different type of mutations, namely base substitutions, frameshifts, deletions and insertions [2, 3].

General outline of the assays

Plasmid pBR322 carries two genes that specify the resistance to ampicillin and to tetracycline. The tetracycline resistance gene is the target gene in the present mutation assay. Plasmid DNA is treated *in vitro* with

chemical mutagens. The chemically modified plasmids are used to transform SOS-induced or non-induced *E. coli* cells. Transformants are selected on ampicilin containing plates. Toothpicking of individual transformants on tetracycline-containing plates allows the isolation of tetracycline sensitive mutants. The mutant plasmids are isolated and the mutations are determined by DNA sequencing.

The tetracycline resistance gene is 1188 bp long and is therefore too long to be conveniently sequenced on a routine basis. We have chosen to limit the analysis of the tetracycline sensitive mutants to a 276 bp long restriction fragment (*BamHI-SalI*). Several criteria were met in this choice: i) the restriction fragment is located in the early part of the gene; ii) *BamHI* and *SalI* are both unique restriction sites in pBR322 which makes both sequencing and cloning easy. Two alternative strategies have been developed involving either plasmid containing lesions within the *BamHI-SalI* restriction fragment (Fig. 1) or randomly modified plasmid. In the latter approach, an interplasmidic recombination assay allowed us to select, among the tetracycline sensitive mutants, those for which the mutations maps within the *BamHI-SalI* restriction fragment [3].

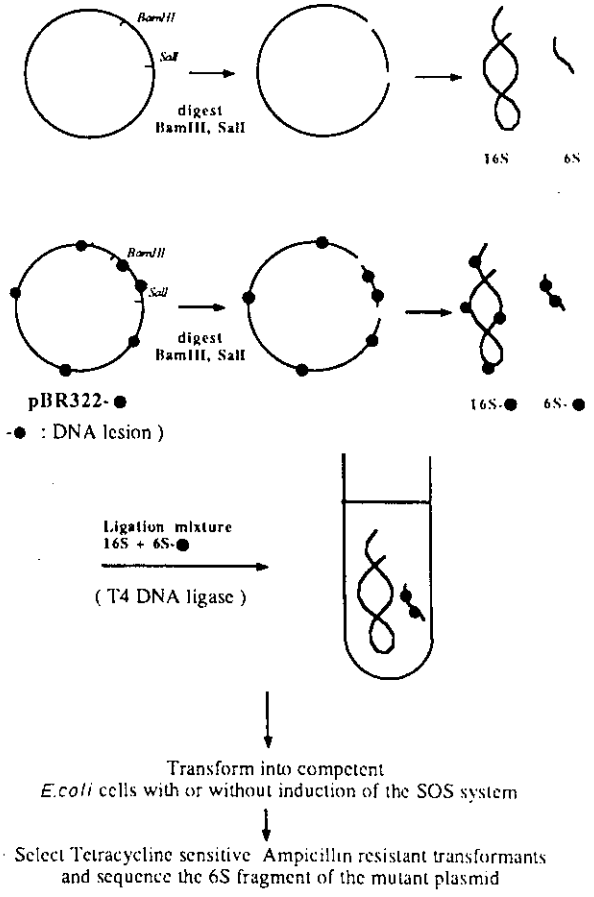

Fig. 1. - Strategy for restriction fragment directed mutagenesis. The black dots represent covalent DNA lesions.

Experimental approach

Chemical modification of the plasmid

Purified plasmid DNA is reacted *in vitro* with chemical mutagens, UV-light or X-rays. Whenever it is possible, radioactively labeled chemicals should be used in order to quantify the extent of reaction. The purification of the chemically modified plasmid DNA (*i.e.* the removal of unbound mutagens) is usually performed by several consecutive extraction of the reaction mixture with an organic solvant (chloroform, phenol, ether, etc.), followed by one or more ethanol precipitations. The extent of reaction can be calculated from the determination of the radioactivity and from the UV absorbance at 260 nm of the DNA (1 unit OD_{260} nm = 50 µg of DNA). The extent of reaction is usually expressed as the average number of lesions per plasmid molecule.

Isolation of chemically modified restriction fragments, cloning and transformation

Chemically modified or unmodified plasmid DNA is digested with *BamHI* and *SalI* restriction enzymes in the buffer specified by the manufacturer. The double digest of pBR322 yields two restriction fragments 276 and 4097 bp long (the fragments are designated 6S and 16S respectively). The two fragments are separated and isolated by sucrose gradient centrifugation. The small fragment (6S) has also been successfully prepared by acrylamide gel electrophoresis (8%) followed by elution [4]. The small *BamHI-SalI* restriction fragment (6S) chemically modified at various extents is recloned in the large unmodified *BamHI-SalI* restriction fragment (16S).

The amount of "reconstructed" plasmid circles was assessed by the relative transformation efficiency of the ligation mixture as compared to native pBR322 plasmid. This number ranges between 5-10% in the control experiment (*i.e.* when non modified 6S fragment is ligated to non modified 16S fragment).

In most cases, efficient mutagenesis requires the induction of the SOS functions prior to the transformation of the bacteria with the damaged plasmid. SOS functions are induced by UV irradiation of exponentially growing bacteria (OD_{700} = 0.2). The cells are centrifuged and resuspended in the same volume of $MgCl_2$ at room temperature. Optimal UV doses for mutagenesis are determined for each strain [2, 3]. For a wild type strain, this dose ranges from 30-80 J/m², for a *uvr* strain it is in the order of 3-6 J/m² corresponding to a 10-20% survival range.

SOS induced or non-induced bacteria were made competent for transformation with a plasmid by means of a classical $CaCl_2$ treatment [5].

Selection and sequencing of the mutants

The wild type tetracycline resistance gene of pBR322 confers the resistance to more than 60 µg/ml of tetracycline. The medium for the selection of tetracycline

sensitive mutants contained either 40 μg/ml or 20 μg/ml of tetracycline. The two concentrations of tetracycline were used to differentiate between "tight" and "leaky" mutants. "Leaky" mutants grow on plates containing 20 μg/ml of tetracycline but not on plates containing 40 μg/ml of tetracycline. "Tight" mutants are unable to grow on either concentrations of the antibiotic.

When tetracycline sensitive mutants are isolated from randomly modified plasmid DNA we use an inter-plasmidic recombination assay to map the mutation as described in Bichara and Fuchs [3]. The mutant plasmids are sequenced according to the method of Maxam and Gilbert [4].

DNA modification spectrum

It is of interest to be able to compare for a given target DNA sequence the mutation spectrum with the DNA modification spectrum (*i.e.* the distribution of the lesions along the DNA sequence). We have developed and used two techniques that allow such a determination. Both techniques make use of the sequencing gel technology to separate a nested set of end labelled DNA fragments that are generated either by exonucleolitic or chemical digestion of a DNA restriction fragment containing the lesions to be mapped. The 3'→5' exonuclease associated to the T4 DNA polymerase has been used to map the N-2-acelylaminofluorene (-AAF) adducts [6]. On the other hand, treatment in hot piperidine was used to cleave and to map N-2-aminofluorene (-AF) adducts in DNA [3].

In order to accurately determine a DNA modification spectrum, the average extent of DNA modification should be in the order of one (or preferably less than one) lesion per restriction fragment. DNA molecules with more than one lesion will produce a P^{32} labeled cleavage fragment corresponding to the lesion that is the closest to the end label in the case of the piperidine treatment and to the lesion that is the farest away from the end label in the case of the T4 enzyme digestion. In any case, these lesions will escape the analysis and consequently will not be scored in the modification spectrum.

Digestion with the 3'→5' exonuclease associated with T4 DNA polymerase [6]. - The 3'→5' exonuclease activity associated to T4 DNA polymerase was found to be blocked in the vicinity of the -AAF adducts. This observation allowed us to determine the binding spectrum of the -AAF adducts along a given DNA restriction fragment. The extent of modification with -AAF was chosen such that on the average each restriction fragment contains in the order of one -AAF adduct per strand. The end labelled 6S fragments are digested with the T4 DNA polymerase associated 3→5' exonuclease activity in the absence of dNTPs.

The digestion pattern obtained for the -AAF modified fragments qualitatively follow the chemical cleavage pattern obtained for the guanine residues in the Maxam and Gilbert reaction (Fig. 2). The densitometric scanning of the sequencing gels made it possible to quantitate the reactivity of each individual guanine residue and to construct the DNA modification spectrum of the double

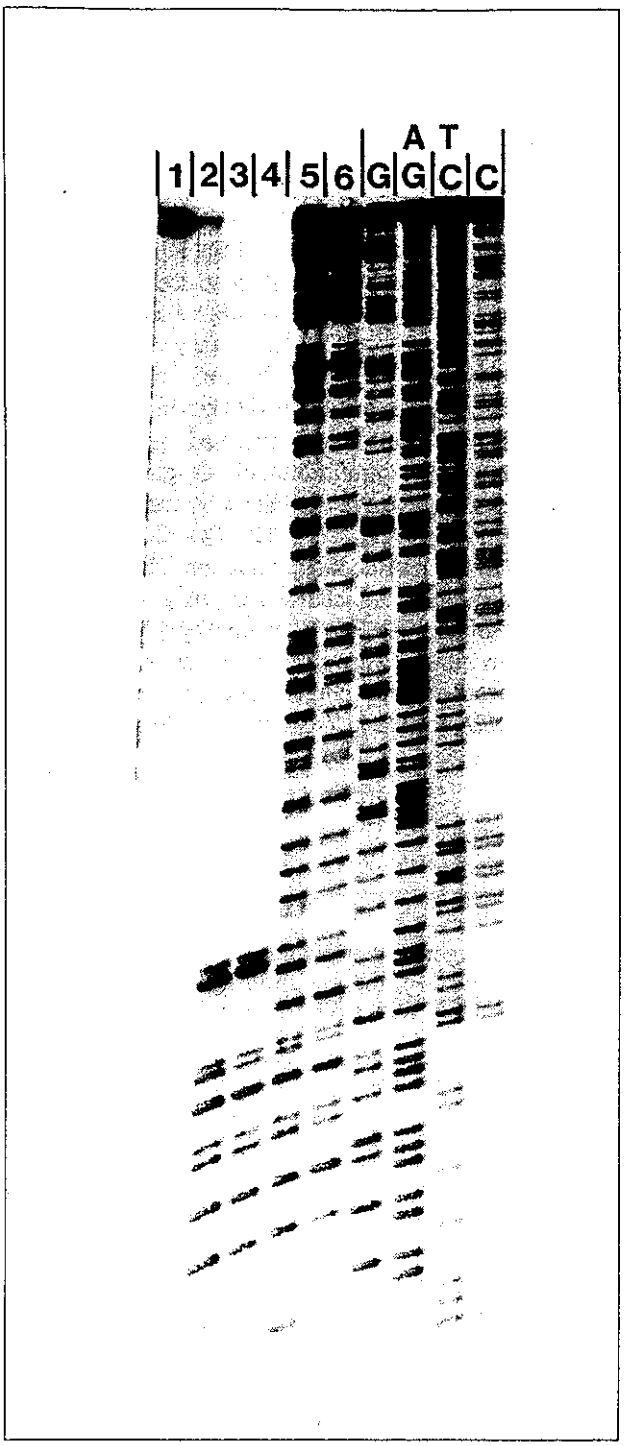

Fig. 2. - Digestion with the 3'→5' exonuclease associated with T4 DNA polymerase. The digestion products are analysed on an 8% sequencing gel. In the autoradiogram that is shown, the double stranded *SalI-BamHI* (6S) fragment is labeled at its 5' *SalI* extremity. Track 2: non-modified 6S fragment digested with 0.9 unit of enzyme. Track 1; 5 and 6: -AAF modified 6S fragment (1.2 AAF adducts / 276 bases) digested with 0, 0.3 and 0.9 unit of enzyme respectively. Track 3 and 4: -AAF modified *SalI-HgiAI* fragment (0.3 -AAF adducts/64 bases) digested with 0.1 and 0.3 unit of enzyme respectively.

stranded *BamHI-SalI* restriction fragment (Fig. 3) [6]. This technique has proven to be very useful in the case of the -AAF lesions since these lesions represent absolute blocks for the T4 enzyme. More recently, the UV-light induced cyclobutane dimers and (6-4) photoproducts were also found to block this exonuclease activity [7]. In the case of both -AF [3] and cis-diamminodichloroplatinum (cisDDP) adducts [8] this technique could not be used since these adducts only slow down the progression of the enzyme without blocking it.

Digestion in hot piperidine. - The determination of the -AF binding spectrum [3] was made possible by the observation that -AF modified DNA fragments undergo a specific strand cleavage reaction when treated in alkali (1M piperidine, 30 min at 90 °C). The alkali sensitivity of several other DNA adducts (aflatoxin B1, benzo(a)pyrene and some UV induced lesions) is known and has been used to study sequence specificity [9-12]. A cleavage pattern qualitatively similar to that obtained in the guanine-specific sequencing reaction is found for -AF modified DNA fragments (see ref. 3 for the data).

Results

Determination of the mutation frequency as a function of the SOS system

With the agents that we have studied so far (-AF, -AAF and cisDDP), the mutation frequency was found to be "inducible". With a plasmid modified at a given extent, the mutation frequency increases as a function of the UV dose given to the bacteria before the transformation step. The mutation frequency reaches a maximum value at a UV dose corresponding to the dose known to fully induce the SOS functions (*i.e.* 30-40 J/m^2 in a wild type strain). In our system, when unmodified plasmid is transformed into SOS induced cells the mutation frequency (untargeted mutagenesis or mutations targeted at criptic lesions present in the DNA sample) was in the order of 6 x 10^{-4} (Table 1). The highest mutation frequencies induced by the chemicals that we have studied were found to be above this background value by about two orders of magnitude.

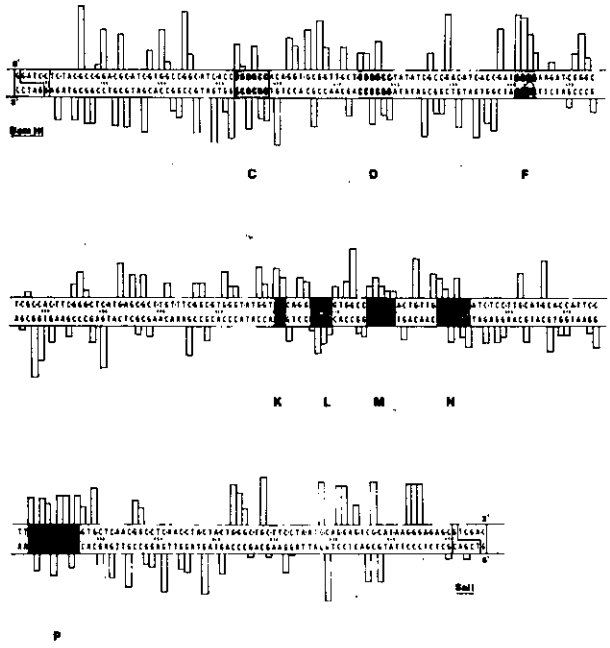

Fig. 3. - DNA modification spectrum by -AAF adducts. The distribution of the -AAF adducts along the 6S fragment is represented by the open bars. The height of the bars represent the relative extent of reaction of the different guanine residues. The areas that appear in grey on the sequence are the sites where -AAF induces frameshift mutation hot spots (A-P).

Determination of the mutation frequency as a function of the number of lesions

A dose dependent increase in the mutation frequency is observed. For the different compounds that have been tested, in non-induced wild type bacteria, the mutation frequency was found to be at least ten times lower than the mutation frequency observed in SOS induced bacteria (Table 1).

Mutation spectra

In order to study mutation spectra induced by chemicals it is recommended to use levels of DNA modification that induce mutation frequencies that are at least ten times above the background. A few hundred of individual tetracycline sensitive mutants have so far been sequenced in our laboratory. Mutants were obtained in different strains (wild type, excision repair deficient: *uvrA*, mismatch repair deficient: *mutU,H,L,S*, deficient in UV mutagenesis: *umuC*) with different mutagenic lesions (-AF, -AAF, cisDDP) [1-3, 8, 13]. The assay was found to respond to frameshift and to base substitu-

Table 1. - *Mutation frequencies in SOS induced and non-induced bacteria*

	-UV	+UV *
no lesion	< 4 x 10^{-4}	6 x 10^{-4}
-AAF (40 / plasmid)	4 x 10^{-4}	130 x 10^{-4}
-AF (111/ plasmid)	20 x 10^{-4}	190 x 10^{-4}
-cisDDP (7.5 / 6S)	20 x 10^{-4}	300 x 10^{-4}

These data were obtained in the wild type strain AB1157.
* The induction of the SOS system was achieved by irradiation of the bacteria before the transformation step at a dose of 30 J/m^2. The extents of modification are given in parenthesis; in the case of cisDDP the adducts are located within the 6S fragment only. The transformation efficiency relative to non modified plasmid DNA was equal to 1% and to 10% for DNA-AAF and DNA-AF respectively. In the case of cisDDP the relative transformation efficiency of this sample has not been determined.

tion mutations. For instance (Fig. 4), -AF lesions induce a majority of single base pair substitutions, whereas -AAF lesions induce frameshift mutations at specific sequences (mutation hot spots). It is clear that the assay will detect (*i.e.*: give rise to tetracycline sensitive mutants) any frameshift mutation within the 6S fragment. As far as the base substitution mutations are concerned, we have obtained 35 different sites within the 6S fragment where a single base substitution gives rise to a tetracycline sensitive mutant (Table 2). We have certainly not yet saturated the base substitution mutation map as for instance several positions where a single base pair substitution produces a stop codon have not yet been scored. Among all the possible base substitution events only AT→CG transversions have not yet been detected. This is probably due to the fact that the mutagens so far studied bind preferentially to guanine residues (except for cisDDP which forms also ApG adducts).

Conclusions

In the present paper, we have described a forward mutation assay based on the inactivation of the tetracycline resistance gene located on plasmid pBR322. This assay involves the introduction, *in vitro*, of known lesions either randomly distributed on the plasmid or localized within a short restriction fragment located in the early part of the tetracycline resistance gene. The plasmid containing the lesions is introduced into bacteria by a transformation step. The SOS functions of the host bacteria can be induced by UV irradiation prior to the transformation step. Using different chemical mutagens this assay was found to be able to detect the different classes of mutagenic events: base substitutions, frameshifts, insertions and deletions.

Since our assay relies on a phenotypic detection of the mutants, many base substitution mutations will not be detected (silent mutations). Therefore, it is hazardous to speak in term of base substitution hot spots, since

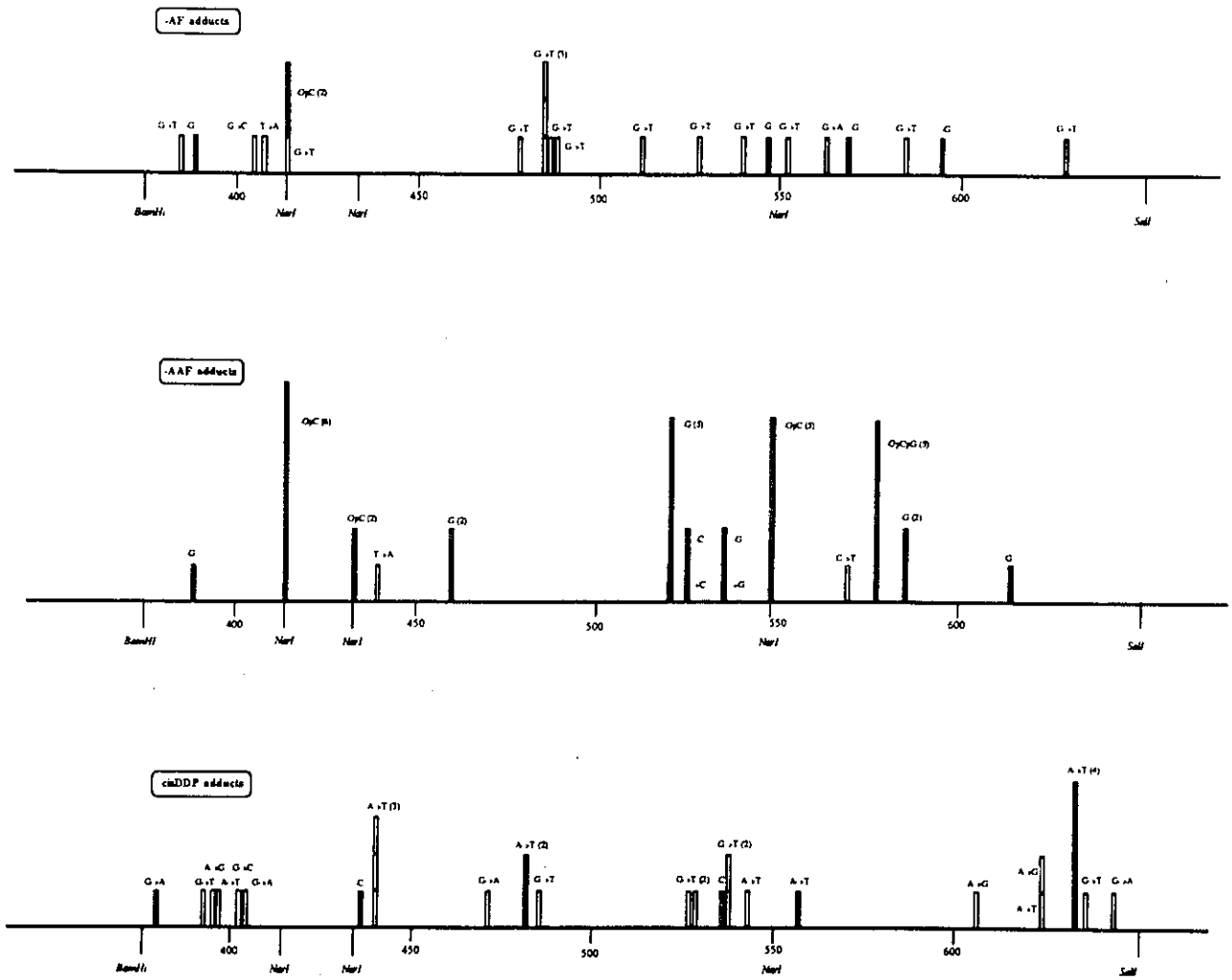

Fig. 4. - Mutation spectra induced by -AF, -AAF and cisDDP adducts within the *BamHI-SalI* fragment in the wild type strain AB1157. For -AAF adducts the spectrum that is shown includes also data obtained in the *uvrA* strain AB1186. Frameshift and base substitution mutations appear as black and white bars respectively. The molecular nature of the mutations is specified as the change that occurred in the coding strand (5'→3' *BamHI-SalI* strand). If a given mutation occurred more than ounce the number of occurrences appears in parenthesis.

Table 2. - *Base substitution mutations that inactive the tetracycline resistance gene*

Nucleotide position	Base change	Amino acid change
385	CG→AT	tyr→ochre
390	GC→AT	gly→glu
404	GC→AT	gly→ser
405	GC→CG	gly→ala
408	TA→AT	ile→asn
414	GC→TA	gly→val
417	CG→AT	ala→asp
426	CG→GC	ala→gly
440	TA→AT	tyr→asn
450	AT→GC	asp→gly
464	GC→TA	val→glu
471	GC→AT	arg →gln
482	TA→AT	phe→ile
485	GC→TA	gly→trp
488	CG→AT	leu→ile
512	GC→TA	gly→cys
513	GC→AT	gly→asp
524	GC→CG	gly→arg
528	CG→AT	pro→his
536	GC→CG	gly→arg
537	GC→TA	gly→val
539	GC→AT	gly→arg
540	GC→TA	gly→val
543	TA→AT	leu→gln
552	CG→AT	ala→asp
557	TA→AT	ser→thr
570	CG→TA	pro→leu
606	TA→CG	leu→pro
615	GC→AT	gly→asp
624	TA→AT	leu→gln
624	TA→CG	leu→pro
633	AT→TA	glu→val
636	CG→AT	ser→ambre
644	GC→AT	gly→arg

such hot spots, if observed, most likely reflect biases introduced by the selection procedure and not specificity related to the mechanisms of mutagenesis. Mutation assays capable to detect all base substitution events [14] are needed if one wants to study base substitution hot spots. In contrast, there is no selection bias in our assay for frameshift mutations. Therefore, frameshift mutation hot spots are of significance and point to specific DNA sequences within which a given mutagen induces a specific structural alteration that is substrate for the mechanisms that fix the mutations (mutation prone sequences as defined in ref. 2 and 6). Two kinds of such sequences were found in the case of -AAF induced mutations (sequences containing runs of guanine residues and short stretches of alternating GC sequences [1, 2, 6, 15, 16]).

More recently, we have used well caracterized tetracycline sensitive plasmid mutants (isolated in the course of the forward mutation assay or constructed by genetic engineering) in reversion assays [16]. This latter approach offers the possibility of easily measuring accurate mutation frequencies at precise loci.

Strains defective in different repair genes (in the excision or mismatch repair pathways) or strains defective in the *umuC* gene have been used to investigate the role of these pathways in the mechanisms of mutagenesis [2, 13].

Since the target gene for the mutation assay is located on a plasmid, premutagenic lesions can be introduced *in vitro* under well controlled conditions. The formation of specific adducts can in some cases be achieved by the appropriate choice of the reactive chemical or by the adjustment of the reaction conditions between the chemical and the DNA. In any case, it is possible to obtain well defined levels of DNA modification and to determine the chemical structure of the adducts. Moreover, lesions can be directed to the sole target gene or within a restriction fragment thereof by the use of *in vitro* ligation procedures. The *in vitro* reconstructed, chemically modified plasmid is introduced into bacteria where the premutagenic lesions are repaired or converted into mutations. This kind of procedure can be referred to as *in vitro-in vivo* protocols. In contrast to *in vivo* mutagenesis protocols, where a whole living organism is treated with a mutagen, these *in vitro-in vivo* procedures will strongly increase the efficiency of the mutagenesis by limiting the toxic effect of the chemical to a very short piece of DNA (*i.e.*: the target gene). In some cases, it is also possible to construct the DNA modification spectrum. The comparison between modification and mutation spectra will greatly facilitate the design of sophisticated mutation experiments involving a single well defined adduct located on a single position in a genome (site directed mutagenesis).

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