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Biological Properties of Single Chemical–DNA Adducts: A Twenty Year Perspective

James C. Delaney and **John M. Essigmann***
Departments of Chemistry and Biological Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139

Abstract

The genome and its nucleotide precursor pool are under sustained attack by radiation, reactive oxygen and nitrogen species, chemical carcinogens, hydrolytic reactions, and certain drugs. As a result, a large and heterogeneous population of damaged nucleotides forms in all cells. Some of the lesions are repaired, but for those that remain, there can be serious biological consequences. For example, lesions that form in DNA can lead to altered gene expression, mutation, and death. This perspective examines systems developed over the past 20 years to study the biological properties of single DNA lesions.

1. Introduction

Twenty years ago, the field of site-specific mutagenesis by DNA lesions was at the transition between its infancy and early adolescence. At that time, the Editor of this journal asked one of us to write a perspective (1) that became the first article published in *Chemical Research in Toxicology*. Now that the field has reached maturity, the Editor has once again asked us to take stock of it. He has asked us to look back and assess the accomplishments of the entire field and then to look forward to see what might be expected as we gaze down the path ahead. The perspective is not meant to be a comprehensive review because, indeed, the field is now of such size that it is best reviewed in a fragmented way (see, for example, ref 2). Rather, we look back with 20/20 hindsight to identify past milestones that, in our opinion, have pushed the field ahead technologically or conceptually. As shown in Figure 1, advancements made by synthetic chemists, molecular biologists, and biophysicists have made possible the testing of hypotheses regarding lesion genotoxicity, mutagenicity, and repair. We apologize in advance to those who have made important contributions that did not fit into the boxed areas in Figure 1, which we describe below, or were not mentioned. We have been very selective and, at times, seemingly arbitrary in our choice of topics. Lastly, we look ahead and make guesses as to the new areas in which this field could contribute in the future.

Site-specific lesion mutagenesis, bypass, or repair experiments use site-specifically modified oligonucleotides or genomes to monitor the fates of a chemical lesion situated at a known position within DNA or RNA. The fates studied are those evident after the lesion has been encountered by a polymerase (e.g., mutation and bypass) or repair protein (e.g., suppressed mutation and toxicity) in vivo. This field began as a logical offshoot of the fields of carcinogenesis and drug development, fields in which DNA damage is often a central concern. Most carcinogens and many drugs are or generate electrophilic intermediates that damage many sites in DNA. The resulting DNA lesion or adduct population is often so vast that it is difficult to determine with certainty which specific chemical modification of DNA was responsible for

*To whom correspondence should be addressed. Tel: 617-253-6227. Fax: 617-253-5445. jessig@mit.edu.

mutagenic or lethal events. Consequently, about a quarter of a century ago, workers started engineering the genomes of viruses and plasmids to contain, at specific genome sites, the DNA adducts caused by genotoxins. The genomes were replicated in cells, and the output progeny was analyzed to determine the type, amount, and genetic requirements for mutagenesis by the specific lesions. The objectives of the work were 2-fold: first, to rank order the toxicity and mutagenicity of DNA lesions and, second, to pinpoint the specific lesions that gave rise to the mutational spectra observed in the wake of DNA damage to tissues. In this perspective, we describe the advances made by groups concerning the synthesis of site-specifically modified oligonucleotides, as well as the methodology to track specific lesions of interest in vitro and in vivo.

2. Synthesis of Oligonucleotides Containing Site-Specifically Modified Bases

2.1. Synthesis of Modified Phosphoramidites

The current state of the art in DNA synthesis allows one to envision building an entire genome containing the damage of interest through solid-phase chemical synthesis (3,4). However, because machine synthesis has not yet achieved the speed and accuracy that natural DNA replication can provide, from a technical and purity standpoint, it is easier to tap into an existing genome by splicing in a short piece of purified and characterized DNA containing the damage of interest located at a unique site. The first oligodeoxynucleotide containing site-specific damage contained O^6-methyldeoxyguanosine in a tetramer made by manual solution-phase phosphotriester chemical synthesis (5). Today, thanks to the chemistry of the Caruthers' group (6–8) and the instrumentation of Ogilvie (9) and Hood (10), automated solid-phase DNA synthesis using phosphoramidites has become the industry standard, and many phosphoramidites containing biologically interesting base modifications are now commercially available. The discovery of new chemical reactions, such as the Buchwald–Hartwig palladium-catalyzed cross-coupling aryl amination reaction (11–14), provides a route for synthetic chemists in the field of chemical toxicology to make DNA adducts stemming from industrial and dietary mutagens. Much pioneering work in the field was and continues to be performed by synthetic chemists, who work out conditions whereby the modified base in the phosphoramidite containing the customized damage is stable to not only the reagents involved in chemical synthesis but also to those involved in deprotection of the oligonucleotide. A short list of notable chemists in the modified DNA phosphoramidite field, who have made oligonucleotides with an emphasis on solving biological problems, is presented below.

Francis Johnson has made DNA containing 8-oxo purines (15), 8-aminoguanosine (16), ethano- and ethenocytidine (17,18), ethenoguanosine (19), synthetic abasic sites (with Arthur Grollman) (20), guanosine adducts of acrolein (21), amino-naphthalene and aminofluorene (22), benzo[*a*]pyrene (23), the dietary mutagen PhIP (24), and tamoxifen (with Shinya Shibutani) (25). Shinya Shibutani has made DNA using guanine phosphoramidites bearing methyl, phenyl, ethyl, and tamoxifen adducts (25–29). Lawrence Marnett has made the deoxyguanosine malondialdehyde adduct (30). Lawrence Sowers has made DNA with pyrimidines oxidized at the C5-methyl or C5 ring positions (31–34). Jean Cadet has made DNA containing the 5′,8-cyclopurines (35), cyanuric acid (36), and the thymidine oxidation products 5-hydroxy-5-methylhydantoin (37), 5-carboxyuridine (38), and thymidine glycol (39). Shigenori Iwai synthesized oligonucleotides containing both stereoisomers of thymine glycol (40–42), as well as pyrimidine (6-4) pyrimidone, Dewar, and cyclobutane pyrimidine dimers (43–46). The incorporation of radical precursors into DNA by Bernd Giese at the C4′ sugar position (47–50) and by Marc Greenberg at the C1′ and C4′ sugar positions (51–54) and at the corresponding C5-methyl, C5, and C6 pyrimidine ring positions (55–62) has furthered our understanding of the products and mechanism of how site-specific radical formation may lead to DNA strand breaks, sugar and base damage, tandem lesions, and cross-links, which may themselves be used for further biological studies. Marc Greenberg has also made DNA

containing bona fide formamidopyrimidine (FAPY) guanine and adenine lesions (63,64), oxidized abasic sites (65,66), and dihydro-5-hydroxythymidine (67). Thomas Harris has made the DNA acrolein adducts of guanine (68) and epoxybutene adducts at N1 of inosine (69). Carmelo Rizzo has made deoxyguanosine lesions containing etheno and hydroxyethano damage (70), as well as the C8 and N^2 derivatives from the dietary mutagen IQ (71,72). John-Steven Taylor has made the *cis*- and *trans-syn* photoproduct lesion between vicinal and nonadjacent thymidines (73–76). We have made DNA containing 5-hydroxy derivatives of cytidine and uridine (77) and the complex psoralen-thymidine monoadduct (78). Paul Hopkins has used a phosphoramidite strategy for making DNA interstrand cross-links between guanosines joined together by a common exocyclic nitrogen (79). Donald Jerina has made DNA bearing polycyclic aromatic hydrocarbon adducts of adenosine and guanosine (80–84) and, with the late Anthony Dipple, studied the effect of sequence context and stereochemistry on mutagenicity (85–92). The late Bea Singer was successful in making ethano-deoxyadenosine (93) and hydroxymethyl-etheno-deoxycytidine (94), and she, along with Dezider Grunberger, contributed enormously to the scholarly foundation of the field of alkylation chemistry of nucleic acids (95).

2.2. Postsynthetic Modification of DNA Bearing a Convertible Nucleoside

In contrast with the preceding section dealing with phosphoramidites that are fully equipped with their lesion, Mark Matteucci (96), Gregory Verdine (97–100), Peter Swann (101–103), Constance and Thomas Harris (104–107), and Roger Jones (108) pioneered the convertible nucleoside approach to site-specifically replace the exocyclic groups of DNA bases postsynthetically with a variety of *N*-, *O*-, and *S*-substitutions. The phosphoramidites containing postsynthetically displaceable leaving groups are commercially available, and researchers in the field can now, in many cases, easily gain access to their modified base of interest by simply displacing the leaving group in the postoligomerized oligonucleotide with an amine, alcohol, or thiol, rather than having to do a total synthesis of each phosphoramidite of interest (e.g., displacement of the O^6-guanine leaving group with methanol will give the carcinogenic lesion O^6-methyldeoxyguanosine) (102). Skilled synthetic chemists are still needed for the construction of complex nucleophiles containing specific stereochemistry, and the Harris' have employed this umpolung technique to make several aromatic and aliphatic DNA lesions (109–114). Indeed, they made such diverse purine adducts as the polycyclic aromatic hydrocarbon benz[*a*]anthracene (105), styrene oxide (104), butadiene diol epoxides (109), and interstrand cross-links (115) in quantities large enough for Michael Stone to obtain NMR structures (116–120). This postsynthetic route was perhaps the only way to obtain DNA containing the malondialdehyde adduct of adenosine, which Carmelo Rizzo has made in addition to the guanosine adducts of malondialdehyde and *trans*-4-hydroxynonenal (121, 122). We have used this technique to make a furfuryl-N^2-deoxyguanosine adduct (123) and, using a nonconventional convertible nucleoside, 5-guanidino-4-nitroimidazole (124). Isao Saito and Jean Cadet have used one-electron oxidation chemistry on DNA to postsynthetically convert 8-methoxyguanosine to 2-aminoimidazolone (125) and 5-hydroxyuracil to isodialuric acid (126), respectively. Using radical precursors, Jean Cadet and Yinsheng Wang have made C8-guanosine-pyrimidine DNA intrastrand cross-links (127,128). Traditional convertible nucleosides have also been used to isotopically label the exocyclic groups for NMR studies (129,130).

2.3. Postsynthetic Modification of DNA Bearing 8-Oxo-guanosine

While not a convertible nucleoside in the umpolung sense, one can view 8-oxoguanosine (and possibly even guanosine) as an "Achilles heel" convertible nucleoside, in that its low oxidation potential will attract damage to its position when an oligonucleotide is exposed to oxidizing agents. This potential liability is sometimes exploited in that one can choose the oxidant that will form the largest percentage of a sought-after specific lesion and then fish out their lesion

of interest by HPLC or capillary gel electrophoresis purification. Cynthia Burrows was able to gain control over the DNA product distribution of 8-oxoguanosine conversion to spiroiminodihydantoin and guanidinohydantoin by an iridium oxidant using temperature and pH (131). Steven Tannenbaum modulated the distribution of DNA products from 8-oxoguanosine conversion by varying the concentration of peroxynitrite oxidant (132), and this site-specific oxidation technique was used to make oligonucleotides containing oxaluric acid, oxazolone, and cyanuric acid (133). Using photochemical oxidation, Nicholas Geacintov converted a DNA oligonucleotide containing a single guanine to one containing 8-nitroguanine (134). Conversion of a normal base within an oligonucleotide is not limited to guanine and its analogues, since site-specific transformations can be performed using base-specific chemistry, given that the base or reaction site is unique within the oligonucleotide; for example, we have converted an oligonucleotide containing a single thymine to one containing thymine glycol using permanganate oxidation (135), and John-Steven Taylor has converted vicinal thymidines to their (6-4) and Dewar photoproducts using 254 nm irradiation (136).

2.4. Postsynthetic Modification of DNA by Direct Reaction with Electrophile

In certain cases, one can circumvent the need for modified phosphoramidites by allowing an oligonucleotide to react directly with the electrophilic carcinogen or model compound to obtain the desired adduct, which has been performed in high yield with some polycyclic aromatic hydrocarbons. Nicholas Geacintov took this approach to make adenosine adducts of benzo[*c*] phenanthrene, and guanosine lesions of methylchrysene (137,138) in quantities large enough for NMR solution structures to be obtained by Dinshaw Patel, with molecular modeling by Suse Broyde (139,140). Likewise, the preparation of the relatively stable *exo*-8,9-epoxide of aflatoxin B_1 using dimethyldioxirane, devised by Thomas Harris (141), allowed him to make enough of the N7-guanosine adduct (142) for Michael Stone to solve the NMR solution structure (143). Robert Fuchs used the direct reaction technique to synthesize C8-acetylaminofluorene adducts of guanosine, as well as an intrastrand cisplatin ApG adduct (144,145); we and Stephen Lippard made the cisplatin GpG adduct (146). Using DNA containing only one guanosine or adenosine, Shinya Shibutani has made tamoxifen adducts of guanosine and quinone-derived estrogen adducts of guanosine and adenosine (147–151). Ashis Basu used DNA containing a single guanosine to make C8-aminopyrene and nitropyrene lesions (152,153) and targeted adduction of mitomycin C to a specific location (154). Edward Loechler made a nitrogen mustard interstrand cross-link (155), and Paul Hopkins made interstrand cross-links from the anticancer agent BCNU, cisplatin, and nitrous acid (156–158).

2.5. Utility of DNA Bearing Isotopically Labeled and Atomically Mutated Bases

Oligonucleotides containing modifications not directly related to natural or environmental damage can also have a deep impact on the field of biological chemistry in general. Although undamaged, the phosphoramidites made by Roger Jones containing specifically placed multiple heavy isotopes have great utility in solving NMR solution structures of DNA (159–161), in investigating the mechanism of DNA base transformation by oxidative damage (162), and in acting as internal standards for mass spectrometry (163). Natalia Tretyakova has used these products to determine the reactivity (hotspot formation) of bases in varying sequence contexts toward DNA alkylating agents (164–169), as well as to quantify the amount of inter- vs intrastrand DNA cross-links formed by bifunctional alkylating agents (170). Thomas Spratt has made phosphoramidites containing atomically mutated bases to study the mechanism of DNA repair by alkyltransferases (171,172) as well as DNA replication (173,174), while Eric Kool has replaced individual atoms of bases to gain insight into the steric vs hydrogen-bonding requirements of biological processes, such as DNA replication (175–180), DNA recognition by mismatch repair complexes (with Peggy Hsieh and Thomas Kunkel) (181,182), and DNA repair by Fpg and MutY (with Shelia David) (183).

3. In Vitro Studies Using Site-Specifically Modified Oligonucleotides

3.1. DNA Purification, Characterization, and Curvature Assay

Once the site-specifically modified oligonucleotides have been made, they are purified by one or several methods. Oligonucleotides are usually resolved based on charge using anion-exchange HPLC and an increasing ammonium acetate salt gradient or based on hydrophobicity using reversed-phase HPLC and an ion-pairing agent, such as triethylammonium acetate, with an increasing acetonitrile organic gradient. Denaturing high-percentage polyacrylamide gel electrophoresis resolves oligonucleotides based on their mass-to-charge ratio, but practically speaking, smaller failure sequences migrate faster, as is also seen for anion-exchange HPLC. After desalting and characterization by mass spectrometry and/or HPLC of the nucleosides or bases generated from an enzymatic or acid chemical hydrolyzate, respectively, the oligonucleotides can be used directly for biophysical studies, such as NMR, crystallographic, or melting temperature analyses to extract thermodynamic parameters on the helical stability of a lesion. Donald Crothers pioneered yet another biophysical method, which uses the ligation of properly phased DNA duplexes to measure DNA curvature, as bent DNA migrates more slowly through a nondenaturing gel (184,185); he and Stephen Lippard used this to determine that cisplatin GpG intrastrand cross-links bend DNA 40° toward the major groove (186). While Nicholas Geacintov used the assay to show the stereochemical influence of benzo[*a*]pyrene-N^2-guanine derivatives on DNA bending (187–189), the Stone, Hopkins, and Harris groups have used this assay to measure DNA bending induced by propanoguanine opposite a 2 bp deletion, mechlorethamine-N7-guanine interstrand cross-links, and alkane-N^2-guanine intrastrand cross-links, respectively (190–192).

3.2. Cocrystal Structures of DNA Lesions with Replication and Repair Proteins

NMR solution studies of DNA lesions, such as the recently investigated ring-opened aflatoxin-N7-guanine FAPY adduct by Stone and the Harris' (193), are informative in providing data on equilibrating isomers, which are valuable for the structural interpretation behind observed mutational spectra (194). However, the majority of structural studies using site-specifically modified oligonucleotides and DNA polymerases or repair proteins have been obtained using X-ray crystallography, which has shed light on mechanism. Tom Ellenberger's cocrystal structures of DNA containing 8-oxoguanine or a thymine dimer with bacteriophage T7 DNA polymerase reveal how a lesion can be accurately or inaccurately replicated or act as a lethal replication block (195–197); his structure of DNA containing 1-azaribose with AlkA suggests a large 66° induced DNA bending, with base flipping and S_N1-type mechanisms during repair (198). Ellenberger's structures of the human AlkA homologue AAG bound to 1-azaribose or ethenoadenine lesions maps out a route for base flipping and excision, providing insight into the mechanism of discrimination between damaged and normal bases (199,200). Sylvie Doublié and Susan Wallace captured stalled complexes of bacteriophage RB69 replicative DNA polymerase with abasic sites in the active and exonuclease sites, and their structure with thymine glycol shows the C5-methyl destacking the 5′-base, providing a rationale for why the lesion is not highly mutagenic and why adenine can be inserted opposite the glycol but cannot be extended further (201–203). Samuel Wilson's cocrystals of pol β with DNA containing 8-oxoguanine, abasic sites, and benzo[*c*]phenanthrene-N^2-guanine in gapped structures provides mechanistic data on dNTP insertion and deoxyribose phosphate lyase repair (204–206). Lorena Beese's cocrystal of DNA bearing benzo[*a*]pyrene-N^2-guanine with the high-fidelity *Bacillus* DNA polymerase shows that the adduct situated in the minor groove greatly distorts the contacts between the DNA and the polymerase (207); additionally, her structural snapshots of O^6-methylguanine with the polymerase reveal how an opposing C and T can evade proofreading, and her structures, obtained with Paul Modrich, of MutSα bound to O^6-methylguanine:T, G:T, G:U, and a one base loop shows all lesions to be recognized similarly by the complex (208,209). Gregory Verdine's cocrystal structures of DNA containing 8-

oxoguanine with hOGG1 or catalytically inactive MutM, of trapped DNA repair intermediates with MutM or EndoIII, and of engineered adenine-MutY and other DNA–protein disulfide cross-links provide a framework for understanding how lesions are recognized, the step by step process by which excision of the lesions occurs during repair, as well as how a repair enzyme can find a single lesion on a vast landscape of unmodified DNA (210–218). John Tainer's cocrystal structures of DNA containing uracil and abasic sites with human uracil-DNA glycosylases show how the enzyme can flip the base out of the helix and into the active site, by insertion of an amino acid residue from the minor groove with phosphate backbone compression, and how base excision repair is initiated (219,220). His structure of endonuclease IV complexed with an abasic site, obtained with Richard Cunningham, reveals a 90° DNA kink, with both the abasic site and its partner flipped out of the helix, thus promoting specific insertion and endonucleic cleavage of the abasic site within the active site pocket (221); a kinked DNA structure was also seen with the APE1 structure (222). John Hunt's crystal structure of 5′-T(1-methyldeoxyadenosine)T-3′ with AlkB shows the alkylated base to be flipped out and also provides a rationale for binding and repair of 1,N^6-ethenoadenine, due to a small packing void adjacent to the 1-methyl group (223).

3.3. In Vitro Lesion Replication Studies

In many cases, the well-characterized DNA or RNA can be used directly or is ligated into a larger construct for in vitro lesion replication or repair studies. Such replication studies (mutagenicity and bypass) are straightforward, since the extension of an annealed 5′ ^{32}P-labeled primer past a template lesion in the presence of nucleotide triphosphates and the polymerase under investigation can be monitored by PAGE resolution of ^{32}P-labeled extension products. This method can also be used to determine the ability of a polymerase to incorporate modified nucleotide triphosphates. Myron Goodman established a gel assay for determining the kinetic parameters for the efficiency of nucleotide insertion (V_{max}/K_m) opposite a base, which determines the preference of different DNA polymerases for normal or modified dNTPs opposite normal or modified template bases (224–227). This advance led he and Roger Woodgate to show the relative impact of pol III, pol IV, pol V, and accessory proteins in trans-lesion synthesis (TLS) past photodimers and abasic sites, which led to a model for SOS lesion-targeted mutagenesis (228,229). Graham Walker and we used such an assay to determine that *Escherichia coli* pol IV and its mammalian orthlogue, pol *κ*, are more efficient in accurate TLS past the model nitrofurazone-derived lesion N^2-furfuryl-dG than past an undamaged template base (123). Using a variety of lesions and DNA polymerases, the Guengerich laboratory has performed several presteady-state (rapid quench) experiments, in addition to steady-state measurements, kinetic model fitting, and obtaining crystallographic data on lesion-polymerase-dNTP ternary complexes, to determine how individual steps in polymerization contribute to dNTP incorporation opposite the lesion, such as nucleotide binding, conformational change, phosphodiester bond formation, the efficiency of base pair extension, and the contribution of accessory proteins (230–244). Grollman and Shibutani, in addition to determining the traditional steady-state kinetic parameters for nucleotide incorporation, devised an ingenious in vitro method allowing for PAGE resolution of base substitutions and frameshift deletions resulting from trans-lesion extension of a labeled primer by DNA polymerases in the presence of equimolar triphosphates, thus screening for mutation frequency and specificity in a single-tube reaction (245–257). Indeed, Shibutani's two-phase 20% PAGE modification allowed him to find the miscoding specificities of oxidized and alkylated purines by a variety of *E. coli* and mammalian DNA polymerases (26–28,147,149,151,258–263).

3.4. In Vitro Lesion Repair Studies

Repair studies performed in vitro are straightforward when a base excision mechanism is involved, since a cleavage event (either direct or induced chemically from the newly generated abasic site) can be followed easily by denaturing PAGE resolution of bands from a 5′ ^{32}P-

labeled substrate. However, lesions repaired by a direct reversal mechanism, such as O^6-methylguanine by Ada or Ogt and 1-methyladenine by AlkB, will not produce cleavable substrates. In this case, the amount of alkylated and dealkylated oligonucleotide can be quantified by integration of peaks following reversed-phase HPLC resolution of the oligonucleotides and by mass spectrometry (using appropriate controls or standards). One can enzymatically induce cleavage of the repaired, dealkylated DNA if the lesion is situated in a restriction endonuclease site (264), which allows one to follow repair by PAGE resolution of ^{32}P-labeled bands (265). Additionally, Anthony Pegg and F. Peter Guengerich showed that oligonucleotides containing or lacking O^6-alkylguanine can be resolved by PAGE, provided that they are small enough (266).

Susan Wallace has melded in vitro bypass and repair techniques with in vivo analysis to determine the sequences (hotspots) surrounding 8-oxoguanosine that are most likely to be mutagenic and refractory to repair (267). The lesion is flanked by randomized bases, traversed by a DNA polymerase, and digested with MutM (weeding out nonmutagenic 8-oxoG:C pairings), and lesion-bearing strands that survived are PCR amplified and cloned into a vector to determine the nature of the mutation by sequencing; a similar study was done for abasic sites (268). Richard Wood, Peter Robins, and Tomas Lindahl devised an assay to monitor the repair and resynthesis of covalently closed DNA-damaged plasmids in vitro using mammalian cellular extracts and [α-^{32}P]-dNTPs (269), which was used to measure site-specific repair and resynthesis of cisplatin intrastrand DNA cross-links, photoproducts, 2-acetylamino-fluoreneadducts,and5′,8-cyclodeoxynucleoside lesions(270–275). A comparable assay that achieves a similar goal was devised by Aziz Sancar and colleagues (276).

4. In Vivo Lesion Replication and Repair Studies

Lesion mutagenesis, bypass, and repair studies performed in vitro are unequivocal in that one knows exactly which polymerase or repair protein causes the observed effect. One issue, however, with this approach is that one can force a result that may not be biologically relevant; it is important to interpret results with the caveat that there are multiple DNA repair enzymes and multiple bypass polymerases within a cell, all competing for the same lesion. Accordingly, the most direct way to discern which protein may be the most relevant in lesion processing (and validating in vitro results) would be to place a site-specific lesion into a genome, which is allowed to be replicated and repaired in wild-type cells and, in parallel, in cells knocked out or down for, and/or induced for, specific polymerases or repair proteins. Methods for generating such site-specific genomes are described below.

4.1. Construction and Utility of Site-Specifically Modified Double-Stranded Vectors

Most studies on site-specific lesion replication and repair within cells involve the construction of double-stranded or single-stranded vectors bearing the lesion, the choice of which depends on the particular problem that one wishes to address. Double-stranded vectors are ideal for monitoring nucleotide excision repair (NER) or base excision events, since many repair systems require duplex DNA and one has control over what is placed opposite the lesion. However, numerous studies using duplex genomes have shown that many DNA lesions are blocks to DNA replication, strongly favoring the replication of the nonadducted strand and the reduction of mutation signal. Thomas Kunkel has advanced the fields of both protein engineering and site-specific lesion mutagenesis by devising a method for generating single-stranded M13 viral template strands containing uracil by growing phage in dUTPase and uracil-DNA-glycosylase-deficient *E. coli* (277). After priming with a mismatched (or lesion-bearing) oligonucleotide to program the mutation, extension occurs using T7 DNA polymerase to avoid displacement of the original primer (278), and T4 DNA ligase seals the nick prior to transformation into a uracil-DNA-glycosylase-proficient strain; replication in this strain generates abasic sites in the template strand, thus giving the newly synthesized mutagenic (or

lesion-bearing) strand a competitive advantage in growth (279). Many groups have made double-stranded genomes bearing uracils in the nonlesion strand as a strategy for selective amplification of signal from the nonuracilated, lesion-bearing strand (280–290). Indeed, the Marnett laboratory showed that the uracilated (+) strand is 4 orders of magnitude less able to replicate than a nonuracilated (+) strand (284). For TLS and direct reversal of damage studies by methyltransferases, Gary Pauly and Robert Moschel ensured signal from the lesion strand by ligating into their vector a small duplex DNA in which several uracils oppose the lesion-bearing strand, with the generation of a gap after in vitro degradation of the uracils (287,288). Starting with an unmodified duplex plasmid and one containing randomly incorporated uracils, Robert Fuchs constructed a heteroduplex with the lesion in the nonuracilated strand and then degraded the entire opposing strand to obtain single-stranded modified genomes (286).

The synthesis of double-stranded genomes containing lesions may seem straightforward at first glance. Indeed, why not just digest a double-stranded vector with two restriction endonucleases, remove the small excised piece, and ligate a site-specifically modified duplex containing compatible ends? In practice, however, problems plagued this “traditional cloning” approach. Site-specific genetic studies rely on material that is homogeneous, and the common problems one encounters with traditional cloning would be unacceptable; these problems include concatemerization of the ligation components and poor ligation efficiency due to small overhangs. In response to the need for high-quality site-specifically modified genomes, the gapped duplex method was developed and validated. We were able to ligate a 4-mer containing O^6-methylguanine into a duplex vector by denaturing a mixture of two related duplex genomes that had been linearized at different locations and that differed only by the size and sequence of the complementary oligonucleotide to be inserted, followed by reannealing and ligating the tetramer into the 4-base gap (264). Dephosphorylation of the genome linearized distally from the insert site allows for the removal of the complementary wild-type strand and the analysis of single-stranded genomes, while omission of this step generates duplex vectors. We also used a duplexing strategy whereby a single-stranded circular DNA is annealed with the complementary strand from a related linearized duplex to form the gap, into which the modified oligonucleotide is ligated (291).

Robert Fuchs’ use of gapped duplexes to generate lesions in the leading or lagging strand of covalently closed duplex vectors showed that while C8-guanine-acetylaminofluorene (AAF) adducts are 20-fold more mutagenic in the lagging than in the leading strand, 8-oxoguanine exhibits no such preference (292,293). By crippling the nonlesion strand by UV irradiation of one of the linearized plasmids prior to gapped duplex formation, Fuchs showed that the position of AAF affects −2 deletions within the *Nar*I site (294) and −1 deletions within the *Sma*I site, where a delay of replication after accurate insertion of cytosine opposite the guanine lesion may allow time for the formation of a slipped intermediate (295). Fuchs’ strand segregation analysis assay uses genomes containing a 3–4 nucleotide insertion opposite the lesion, allowing one to distinguish (in mismatch repair-deficient cells) TLS from damage avoidance pathways, which circumvent lesion replication (296–299). Among other discoveries, Fuchs used this assay to show that TLS increases from 1 to 70% when AAF is deacetylated (296) and that AAF-induced −2 deletions in the sequence $GCG^{AAF}CX$ can vary 30–50-fold in *E. coli*, depending on X (298). Lawrence Marnett placed a C:C mismatch 3 kb away from malondialdehyde and cyclopropano guanine lesions and showed that the lesions are substrates for NER by tracking template strand utilization (300). Masaaki Moriya and Arthur Grollman used mismatches at multiple locations to delineate the various repair and replication processes for 1,N^6-ethenoadenine and γ-hydroxypropanoguanine by linkage analysis (301,302).

Another strategy for duplex genome construction uses primer extension on single-stranded circular viral templates. Lawrence Loeb and Bea Singer extended primers by one nucleotide using the dNTP of O^4-methylthymine or N^2,3-ethenoguanine, followed by further extension

with normal dNTPs and ligation of the nick (281,303). Thomas Kodadek and Howard Gamper used a primer bearing an internal psoralen lesion to obtain site-specifically modified duplexes (304).

4.2. Construction and Utility of Site-Specifically Modified Single-Stranded Vectors

Single-stranded genomes are ideal for measuring lesion traversal and mutagenesis by polymerases, since vector replication requires trans-lesion synthesis to occur. Such vectors are also ideal for studying lesion repair by proteins that use a direct reversal of base damage mechanism, such as Ada, Ogt, AlkB, and their eukaryotic counterparts, since repair prior to replication yields a normal base that is neither mutagenic nor genotoxic. Such vectors are not well-suited for addressing NER or base excision repair if the oligonucleotide or lesion is excised with equal efficiency regardless of the opposing base inserted by the DNA polymerase, since the mutation would have already been made permanent. However, repair data can be gained if (i) another enzyme can remove the improper base opposite the lesion, giving the polymerase a second chance for nonmutagenic dNTP insertion, and/or (ii) the lesion is excised only when paired with a nonmutagenic base. This scenario describes the MutM/MutY "GO" repair system that Jeffery Miller and Arthur Grollman discovered for 8-oxoguanine (305, 306); the paradigm may possibly exist for other lesions as well. The GO discovery is a classic example combining in vitro experiments using site-specifically modified oligonucleotides with an original in vivo observation of a mutator phenotype in repair-deficient cells (307,308).

A simple method devised by Christopher Lawrence and J. Eugene LeClerc has gained wide acceptance for constructing site-specific lesions within single-stranded viral genomes. A single-stranded circular viral genome is linearized by cleavage within a hairpin using a restriction endonuclease, followed by annealing of a scaffold complementary to the cleaved termini, into which a phosphorylated oligonucleotide containing site-specific damage is ligated, with scaffold removal by heating in the presence of excess complement (309–311). Lawrence and co-workers have used these genomes to study TLS past photodimers and an abasic site in *E. coli* (309–315) and, with Roger Woodgate, explored the effect of Umu proteins and proofreading on TLS past these lesions (316–318). We adopted this method for the rapid construction of genomes containing highly unstable aflatoxin adducts using a uracilated scaffold, which is removed under gentle conditions by the combined activities of uracil-DNA-glycosylase and exonuclease III (319). Masaaki Moriya's versatile shuttle phagemid pMS2 uses the methodology described above for genome construction (but with the scaffold annealed prior to hairpin cleavage) and allows one to study TLS in mammalian (COS-7 simian kidney) cells, as well as in *E. coli*, for a direct comparison of the cellular responses to a lesion in different environments using the same experimental system (320); the scaffold oligonucleotide is removed using the 3′ to 5′ exonuclease property of T4 DNA polymerase, which we have adopted into our protocols, as we found it to be more effective and to nick DNA less than exonuclease III. We also modified the hairpin cleavage construction technique by using two scaffolds, each of which bridges one end of the cleaved vector and modified oligonucleotide insert, but nothing opposes the lesion or its neighbors, thus providing identical ligation efficiencies regardless of lesion bulk or local sequence context, and assurance of signal from TLS events rather than from any residual scaffold (321).

While this perspective deals primarily with *E. coli* and mammalian studies, a method circumventing the need for genome construction altogether was introduced for studying lesion mutagenesis in yeast. On the basis of the discovery of the Sherman group (322,323), the single-strand oligonucleotide-mediated yeast transformation method uses the electroporation of a single-stranded 26–30-mer oligonucleotide into yeast cells to restore a frameshift allele and has been used to study natural, unnatural, and oxidized abasic sites, uracil (which forms a natural abasic site in vivo upon glycosylase repair), a TT photoproduct, and unnatural lesions

(324–327). Sequencing of revertants provides mutation data, and assuming that modified and unmodified control oligonucleotides integrate into the host chromosome equally, bypass data can be obtained by colony counting.

4.3. Assays for in Vivo Mutagenesis, Bypass, and Repair of Site-Specific Lesions

Once the genomes have been synthesized, they are introduced into cells by electroporation, calcium chloride, calcium phosphate, or lipofection methods. For lesion mutagenesis and repair studies, the goal is to get as many initial independent events as possible for statistical robustness, and it is our experience that electroporation is superior to the calcium method for the transformation of *E. coli*.

The in vivo mutagenicity of a lesion is measured by assessing the percentage of each type of base or frameshift mutation at the lesion site. The blocking power that a lesion has over the polymerase that tries to copy past it within the cell (trans-lesion synthesis/lesion bypass) is often scored as a decrease in survival, with genotoxicity measured as a decrease in the ability to form plaques or colonies. All site-specific lesion repair studies performed in vivo involve mutagenesis and bypass assays that indirectly monitor the event. Repair is inferred by a drop in the mutation frequency or an increase in trans-lesion synthesis when genomes assayed in repair-deficient cells are processed in their wild-type complement. A short list of groups making significant contributions to our understanding of how site-specific lesions are processed within cells is presented below.

Moriya and Grollman used a single-stranded vector bearing 8-oxoguanine to show a striking increase in G to T transversions in MutY-deficient *E. coli*, thus confirming elements of the "GO" system in vivo using site-specific mutagenesis (328). Their use of the pMS2/COS-7 system showed differential mutagenesis for propanoguanine, ethenocytosine, ethenoadenine, and (with Nicholas Geacintov) benzo[*a*]pyrene-N^2-dG adducts as a function of the host cell and, for the latter, sequence context and chirality (329–332); differential oligonucleotide hybridization to individual colonies using probe sets specific for all outcomes provides mutation frequency and specificity, and positive clones can be further sequenced, if desired. Moriya and Grollman engineered yet another vector containing many desirable design elements, allowing for the study of lesions placed in the leading or lagging strand of plasmids replicated extrachromosomally in human cells, as well as in *E. coli* (333,334). For mammalian analysis, plasmids from antibiotic-resistant cells are harvested, treated with *Dpn*I to destroy genomes that have not replicated within the host, and can be treated with another restriction endonuclease to inactivate progeny from the strand complementary to the lesion prior to transformation into *E. coli* for mutational analysis. Because this region is mismatched, omission of the endonuclease will allow the amount of replication from each strand to be quantified, thus providing feedback on lesion-induced inhibition of replication. This versatile system has been used to measure the response of ethenoadenine, α- and γ-hydroxypropanoguanine (and related lesions), and a heptanone adduct of ethenocytosine (333–337).

Shibutani's use of the pMS2/COS-7 system allowed him to determine the mutagenicity of α-tamoxifen-N^2-guanine and estrogen-N^2-guanine and -N^6-adenine adducts (150,338,339). This system allowed him and Grollman to explore, within mammalian cells, such diverse lesions and topics as the effect of sequence context on the mutagenicity of N^2-guanine adducts of (acetyl)aminofluorene and C8-adducts of the cooked food mutagen PhIP; the mutagenicity of N^2-guanine adducts of acetylaminonaphthalene, benzo[*a*]pyrene, and 8-aminoguanine; and a head-to-head mutagenic comparison of 8-oxoguanine vs 8-oxoadenine (257,340–347).

R. Stephen Lloyd evaluated the lethality (plaque forming or transforming ability) and mutagenicity (by differential oligonucleotide probe hybridization) of many alkylated lesions,

mainly obtained by the Harris', using the M13/*E. coli* technique of Lawrence (348–357) and the pMS2/COS-7 technique of Moriya (358–363). Some highlights found in *E. coli* were a dramatic effect of chirality and sequence context on the lethality of α-styrene oxide-N^6-adenine, which is a block to pol III in vitro when in a 33-mer but much less so when in an intact M13; the *β*-isomers are neither toxic nor mutagenic (348,350,355). The *S,S* stereoisomer of butadiene-N^2-guanine intrastrand cross-links is more mutagenic than the *R,R* isomer (354); the N1 deoxyinosine adduct resulting from reaction of 1,2-epoxy-3-butene with deoxyadenosine is 90% mutagenic, giving predominantly A to G mutations (356); DNA pol II appears responsible for the mutations caused by butadiene-N^6-adenine intrastrand cross-links (357); and the butadiene-derived N3-deoxyuridine adduct is 97% mutagenic in COS-7 cells, giving C to T and C to A mutations (363).

Hiroyuki Kamiya and Eiko Ohtsuka performed studies on the mutagenicity of several lesions in the c-Ha-*ras* gene in mouse NIH3T3 cells (364–372), and later, Hiroyuki Kamiya and Hiroshi Kasai studied the mutagenicity of 2-hydroxyadenine, the (6-4) T-T photoproduct, and 5-formyluracil in simian COS-7 cells (373–375).

Edward Loechler (376) and Ashis Basu (135,291,377) performed pioneering work in the early days of site-specific mutagenesis while in this laboratory and have gone on to investigate other lesions and biological problems in their independent careers. Loechler has looked at the biological effects within *E. coli* of nitrogen mustard interstrand cross-links (378,379) and of sequence context and stereochemistry of benzo[*a*]pyrene-N^2-guanine adducts (380–389), combining observations from his single adduct studies with molecular modeling (390–396). Basu has investigated N^2-guanine adducts of nitropyrene and mitomycin C and C8-guanine adducts of nitropyrene and ammonia (154,397–401) in *E. coli*. He has also recently investigated N7-guanine adducts of mitomycin C, tandem 8-oxoguanine/abasic site damage, 8-oxopurines, bona fide ring-opened FAPY lesions, and C8-guanine adducts of nitropyrene in COS-7 monkey cells (402–405).

The plot of mutation type and frequency across a gene is called a mutational spectrum. Mutational spectra are usually nonuniform; that is, there are hot and cold spots (406). The reasons for this nonuniformity are not fully understood at the biochemical level, but several reasonable possibilities exist as follows: (i) At any given site, the formation of an adduct may be influenced by neighboring bases. (ii) Adducts in some contexts may be repaired better than in others. (iii) Polymerases may misreplicate adducts more or in a qualitiatively different manner in one context than in others. (iv) A mutation may be more easily selected or detected in some contexts than in others (perhaps because a particular mutant grows better than other clones). The tools of lesion-specific mutagenesis lend themselves well to help quantify the relative contributions of the four possibilities listed above, and one should be cognizant of the risk of generalizing a result obtained in only one sequence context.

Indeed, one of the more interesting applications of genomes containing specific DNA lesions has been the collection of studies aimed at determining the effect of local sequence context on the ways that DNA polymerases or repair proteins recognize lesions. While a comprehensive analysis on sequence context effects is beyond the scope of this perspective, we note a few studies of interest. Wallace generated consensus sequences for hotspot mutagenesis of 8-oxoguanine using randomized flanking bases and in vitro selection (267), and Grollman and Shibutani explicitly generated consensus sequences for acetylaminofluorene and aminofluorene mutagenesis in vivo by constructing individual genomes containing nearly half of the possible 16 nearest neighbors flanking the C8-guanine lesions, and transfecting these into mammalian COS-7 cells; progeny plasmids were treated with S1 nuclease to eliminate signal from unprocessed input DNA prior to their introduction into *E. coli* for mutant screening by ^{32}P-probe hybridization (344). Our group was interested in finding out if consensus

sequences existed in vivo for O^6-methylguanine mutagenesis by the DNA polymerase or if such sequences existed that made the lesion refractory to repair by the methyltransferases Ada and Ogt. We took a nearest-neighbor approach in constructing the requisite 16 individual genomes but quickly realized that processing the data from five cell strains in duplicate would require the analysis of 160 samples. We concluded that a high-throughput method was needed that would allow analysis of the entire output population from each sample. Such a method would obviate the need to sequence over 16000 individual clones or count even more plaque hybridization spots requiring several probe sets to encompass all contexts for mutant identification (312,348). The REAP procedure, which has become widely used in the field, was developed to address the need for such a high-throughput method. This method is described below.

4.4. REAP Assay for Determining the Mutation Frequency and Specificity of Lesions

To overcome the obstacles described above, the approach that we took to obtain the base composition at the lesion site after biological processing involved incorporating our lesion of interest into the cleavage site for a type IIS restriction endonuclease, an enzyme that cleaves with surgical precision a fixed distance away from its recognition sequence. After restriction endonuclease cleavage of the progeny, the newly exposed 5′-phosphate associated with the site that had contained the lesion is postlabeled with ^{32}P. The radiolabeled DNA is then trimmed down to a small size by another restriction endonuclease, gel purified, and digested to 5′-dNMPs, which are resolved by TLC and quantified by PhosphorImagery to obtain the mutation frequency and specificity. The Restriction Endonuclease And Postlabeling determination of mutation frequency (REAP) assay has evolved over the years to its present state, which is depicted in Figure 2 and described in detail with protocols in ref 407. Although Figure 2 depicts progeny from single-stranded DNA being analyzed, the use of PCR to amplify the region that had contained the lesion allows the assay to determine the mutation frequency and specificity of lesions from both single-stranded and double-stranded DNA genomes, which have been passaged through both *E. coli* or mammalian cells. Indeed, the REAP assay can extract data from lesions that have been absorbed intrachromosomally, and mutation data from RNA genomes containing RNA lesions can be obtained after reverse transcription of the lesion site. In addition to point mutations obtained by sampling from the entire progeny population, the nonphenotypic REAP procedure permits simultaneous statistically robust analysis of frameshifts.

Since its inception, the REAP assay has been used to define the coding specificities of over 50 lesions encompassing alkylative and oxidative damage, as well as unnatural atomically mutated bases; the lesions that have been published are shown in Figure 3 (321,408–425). Using REAP, a number of large scale projects have been completed, and the results of those studies are highlighted as follows. First, REAP was used to define with high precision and accuracy the error frequency of the replicative DNA polymerase of *E. coli* as it attempts to copy O^6-methylguanine (408); the polymerase nearly always reads the lesion as if it were an adenine regardless of sequence context. Repair, however, is highly sequence-dependent, with some sequences [e.g., 5′-A(O^6-methylguanine)-3′] displaying conspicuously sluggish repair. Second, the high-throughput nature of the method allowed a host of guanine oxidation products to be analyzed in parallel, leading to the conclusion that these products are more highly mutagenic than 8-oxoguanine (409,411,413,414). Similarly, the ability to study many lesions at the same time allowed characterization of the substrate requirements of the DNA repair enzyme AlkB, which was found to play a powerful protective role against certain alkylated bases, including etheno- and ethano-adenine lesions (412,415,417). Third, the use of REAP with a multiplexed set of cell strains in which various polymerases were expressed in combinations allowed a detailed picture to be constructed of the genetic requirements for lesion replication fidelity of a large array of oxidized DNA bases (419). Fourth, the method has been

used to probe the fidelity of replication of a set of gradually expanded nonpolar thymine analogues (where both oxygens are simultaneously replaced by H, F, Cl, Br, or I); these studies provide basic data that help explain which structural features of normal DNA bases are critical determinants of replication fidelity and evolutionary adaptability (416).

While designed primarily for the generation of in vivo results on lesion mutagenicity, an in vitro version of REAP can be a powerful tool to assess lesion integrity after genome construction or after exposure to specific chemical or enzymatic environments (412). After a scaffold is annealed opposite the lesion in a single-stranded genome (duplex genomes would need no such scaffold), the material is fed into the assay at the *Bbs*I digestion step, with the ultimate resolution and quantification of the original modified 5′-dNMP from its 5′-dNMP conversion, degradation, or contamination products. As one example, REAP was used to study the Dimroth rearrangement of 1-methyladenine to 6-methyladenine (412). One can envision such an in vitro assay for monitoring the direct reversal repair of alkylated oligonucleotides, such as O^6-methylguanine by Ada, Ogt, or O^6-methylguanine-DNA-methyltransferase or 1-methyladenine by AlkB or its human homologues. By tracking the strand opposite the lesion, one may eventually obtain point and frameshift mutations induced by chosen DNA polymerases.

4.5. CRAB Assay for Determining the Polymerase Bypass Efficiency of Lesions

The ability to measure mutagenesis in vivo is only half the story. Indeed, lesions that are not mutagenic to the cell can still be extremely toxic (412,417). Traditional experiments measure the ability of a lesion to hinder DNA replication by a reduction in plaque or colony formation, with respect to a nonlesion control, after immediate plating of transformed cells. Oftentimes, however, the transfection efficiency of cells, be they *E. coli*, mammalian, or yeast, is variable in practice. To address this problem, the Competitive Replication of Adduct Bypass (CRAB) assay was developed (Figure 2), in which a lesion-bearing genome is mixed with a nonlesion internal standard genome prior to transfection. In the initial version of the assay, plaques from lesion-induced point mutations would be dark or light blue, while the internal standard would be clear (412). A drop in blue plaques with respect to clears would indicate blocks to trans-lesion replication. The work also showed that such survival assays based on immediate plating of the transformation mixture can underestimate the blocking power of a DNA lesion; indeed, a lesion can be a block to replication, but if it is eventually traversed prior to host destruction of the vector, a colony (nonblocking “digital” signal) will form. Analysis of progeny after growth of the mixture in liquid culture, where both lesion and internal standard genomes can compete, gives a truer dynamic “analog” signal for lesion bypass determination. Two limitations of the phenotypic version of this assay were that many plaques had to be counted manually, and lesions that generate frameshifts would artificially decrease the bypass signal. To overcome these obstacles, the CRAB assay for determination of lesion bypass quickly evolved to its present state, depicted on the left-hand side of Figure 2 (407). Salient points are (i) that the proportion of signal from lesion and competitor genomes grown in liquid culture is obtained using REAP methodology but with primers that amplify lesion and competitor progeny equally; (ii) the stored progeny from one sample can be analyzed for both bypass and mutagenesis; (iii) the same input mixture containing lesion and competitor is used for an entire panel of repair- and polymerase-deficient and -proficient cell strains, instilling confidence that signal changes result from cellular variables and not from variations in the input formulation; (iv) the input asymmetry of lesion to competitor provides a reproducible, quantifiable signal for strongly blocking lesions, while an equal mixture would do so for nonblocking lesions.

A number of discoveries have been made using the CRAB assay for lesion bypass in vivo. As a first example, the replication of a series of bases possessing small alkyl functionalities provided a detailed picture of the features of lesion structure that allow polymerase bypass in

vivo and indicate that some are well-repaired (412). These studies complemented the REAP results described above. In addition, replication of lesion-containing genomes in cells with known defects in repair (e.g., AlkB) or altered replication states (e.g., different SOS polymerases expressed) defined substrates for repair and for specific polymerases. It was found, for example, that certain replication-inhibiting etheno lesions, which form as a consequence of inflammation, are good substrates for AlkB in vivo (415); hence, the absence of AlkB results in severe genotoxicity. As a second example, it was demonstrated that the potent replication block of 1,N^6-ethanoadenine, a major adduct formed by the antitumor agent BCNU, is relieved in AlkB-proficient *E. coli* (417). This discovery led to in vitro characterization of the mechanism of the "repair" reaction, which was found to be unique. The repair enzyme, using an oxidative route, converted the highly toxic cyclic adduct into a linear chain that was apparently readily bypassed in vivo.

In vitro biochemical studies, such as that of AlkB on ethanoadenine, nicely complement in vivo findings, especially for lesion repair. The unsaturated derivative of ethanoadenine, 1,N^6-ethenoadenine, is a case in point. Using ESI-TOF mass spectrometry on site-specifically modified oligonucleotides treated with AlkB, it was possible to observe the accumulation of intermediates and product along the pathway of 1,N^6-ethenoadenine repair by the enzyme. As was the case with ethanoadenine, it was possible to delineate the reaction path, which involved, in this case, direct reversal of base damage by epoxidation of the etheno double bond, followed by epoxide hydrolysis to form a glycol, which is removed as glyoxal, with restoration of the adenine within the oligonucleotide (415). Yinsheng Wang has recently adopted and adapted the CRAB and REAP assays, using a mass spectrometry platform for quantifying the genotoxicity and mutagenicity of a guanine-cytosine intrastrand cross-link in *E. coli* (128).

5. Future Directions

5.1. Intrachromosomal Probes for Mutagenesis

To look to the future, it behooves us to reflect on the past, picking up on areas that were pursued incompletely or neglected. Site-specific mutagenesis of DNA lesions has been performed mostly using an extrachromosomal approach, whereby an oligonucleotide is ligated into a single- or double-stranded vector containing an origin of replication, which is replicated independently of the host genome. Many important discoveries have been made using extrachromosomal probes; however, these vectors generally replicate more quickly than host chromosomes. They may, therefore, underestimate the effect of DNA repair on damage within a host chromosome, since the vector lesion site encounters a DNA polymerase more quickly, reducing time for repair, which is measured as an increase in lesion bypass (toxicity) and/or a decrease in mutation. On the other hand, mammalian chromosomes have a compacted nucleosome structure, which may shield the lesion from repair, and data from the more repair-accessible vector may overestimate the effect of DNA repair on damage within a host chromosome. Also, it may remain unknown if there are differences between a vector and a host chromosome in the interaction of a lesion with the DNA polymerase holoenzyme and its accessory proteins. For reasons detailed above, intrachromosomal probes may be the most biologically relevant system to study replication and repair of DNA lesions. We and the Guengerich laboratory performed such experiments by transfecting vectors containing site-specific O^6-methylguanine, O^4-methylthymine, or 1,N^2-ethenoguanine lesions into Chinese hamster ovary cells deficient in methyltransferase or nucleotide excision repair, whereby the vector could not replicate within the host unless it integrated into the host chromosome (426–428). Intrachromosomal analysis of mutations should be a more widely used tool.

5.2. RNA Repair of Site-Specific Lesions in Vivo

The discovery that 1-methyladenine in RNA can be repaired by AlkB suggests an intriguing question and poses a significant research opportunity (429,430). Some human transcripts are both long in size and long-lived temporally; examples include those coding for the muscle protein titin (34,350 amino acids) or for the β-and γ-globin transcripts, which have half-lives between 17 and 80 h, depending on induction (431,432). After the large energy commitment to make such transcripts, it would be disadvantageous if chemical damage rendered an RNA species unreadable or misreadable. It seems reasonable to assume that the cell may use RNA repair as a defense against RNA adducts. An in vivo system in which RNA lesions site-specifically incorporated into biologically active RNA species would allow the hypothesis that RNA repair exists as a physiologically relevant process to be tested.

5.3. Kinetics of Site-Specific Lesion Replication and Repair in Vivo

The biological studies to this point on DNA lesions have defined the mutagenic and toxic properties of lesions. In the future, once the properties of the lesion are established, one could use the lesion as a probe to define the properties of biological systems, such as the kinetics of replication and repair, in vivo. For example, a lesion of predefined mutagenicity and toxicity could be introduced at several sites in a genome, and each site would be ever more distal to an origin of replication. Lesions distal to an origin or downstream from an impediment such as a second lesion or a secondary structural element might be expected to show lower mutation frequencies and lower toxicities than lesions that are more proximal to the origin, due to the increased time that they have for being repaired prior to being replicated. Using the highly quantitative methodology that now exists, the timing and hence the kinetics of replication and repair could be clocked in vivo by using mutagenic and toxicity end points. Such studies would help to establish a more detailed, granular image of the responses of cells to stress than exists at present.

5.4. Translational Aspects of Site-Specific Mutagenesis Studies

In any field, it is critical to identify where possible the translational elements that relate most directly to improvement of the human condition. Lesions of anticancer drugs kill cells, but they also can cause mutations that can engender second tumors in cancer patients treated with chemotherapy. Studies of lesion mutagenesis have helped and can continue to help the drug development process, as shown in studies by Yarema et al. (433,434). Additionally, in work by Loeb and co-workers, lesions of established mutagenicity have been used to dope the nucleotide precursor pool of cells attempting to replicate HIV. The viral genomes accumulated mutations at an accelerated pace that apparently caused the virus to exceed the error threshold for viability. This work led to a novel antiviral therapy and opened a field termed “lethal mutagenesis”. Finally, lesion-specific mutagenesis has and will continue to be of central importance to field workers who need biomarkers predictive of carcinogenic risk. For example, the work of Thomas Kensler and John Groopman that established the aflatoxin-N7-guanine adduct in urine as an excellent biomarker for hepatocellular carcinoma risk in human populations benefited from and is mechanistically anchored to genetic studies showing that the aflatoxin adducts are indeed progenitors of the types of genetic change that occur in people with clinical disease (194,435).

Twenty years ago, it would seem out of the question to process more than a few lesions of interest in vivo, yet alone in different sequence contexts and different cell strains. The progress described herein has increased the productivity of the field by several orders of magnitude, and fundamental discoveries on how cells respond to damage are being made at a staggering rate. It is clear that this field is robust and has a bright and dynamic future. It will be exciting to see what the next 20 years have in store.

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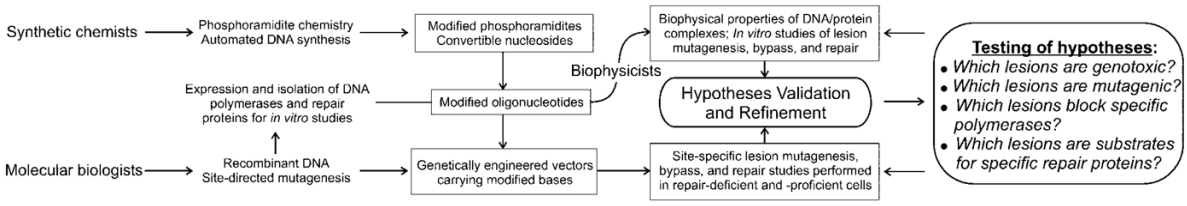

Figure 1.
Evolution for understanding polymerase and repair contributions to lesion mutagenesis and bypass.

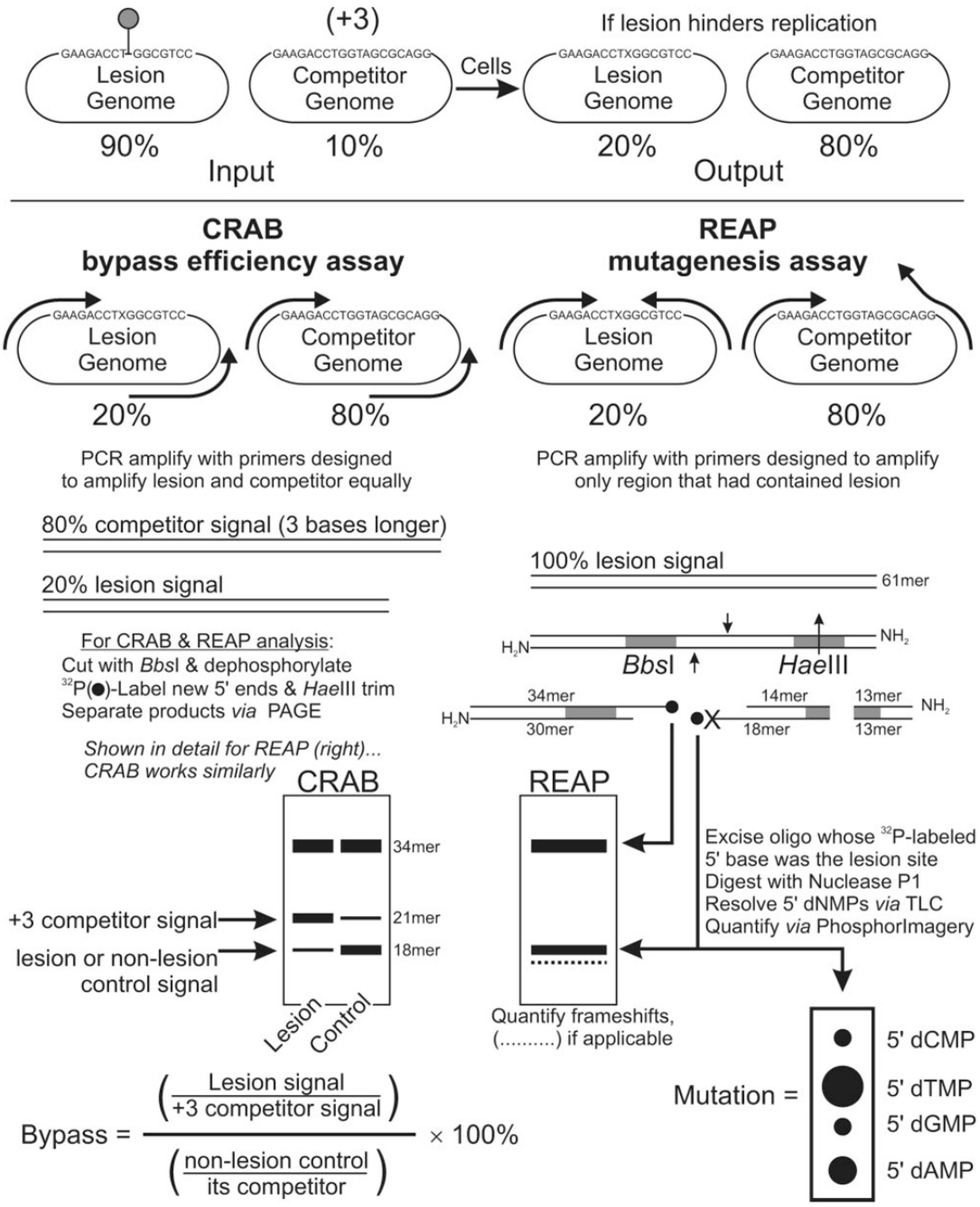

Figure 2.
REAP assay for determining the mutation frequency and specificity of lesions (right) and CRAB assay for determining the replication blocking power of lesions (left).

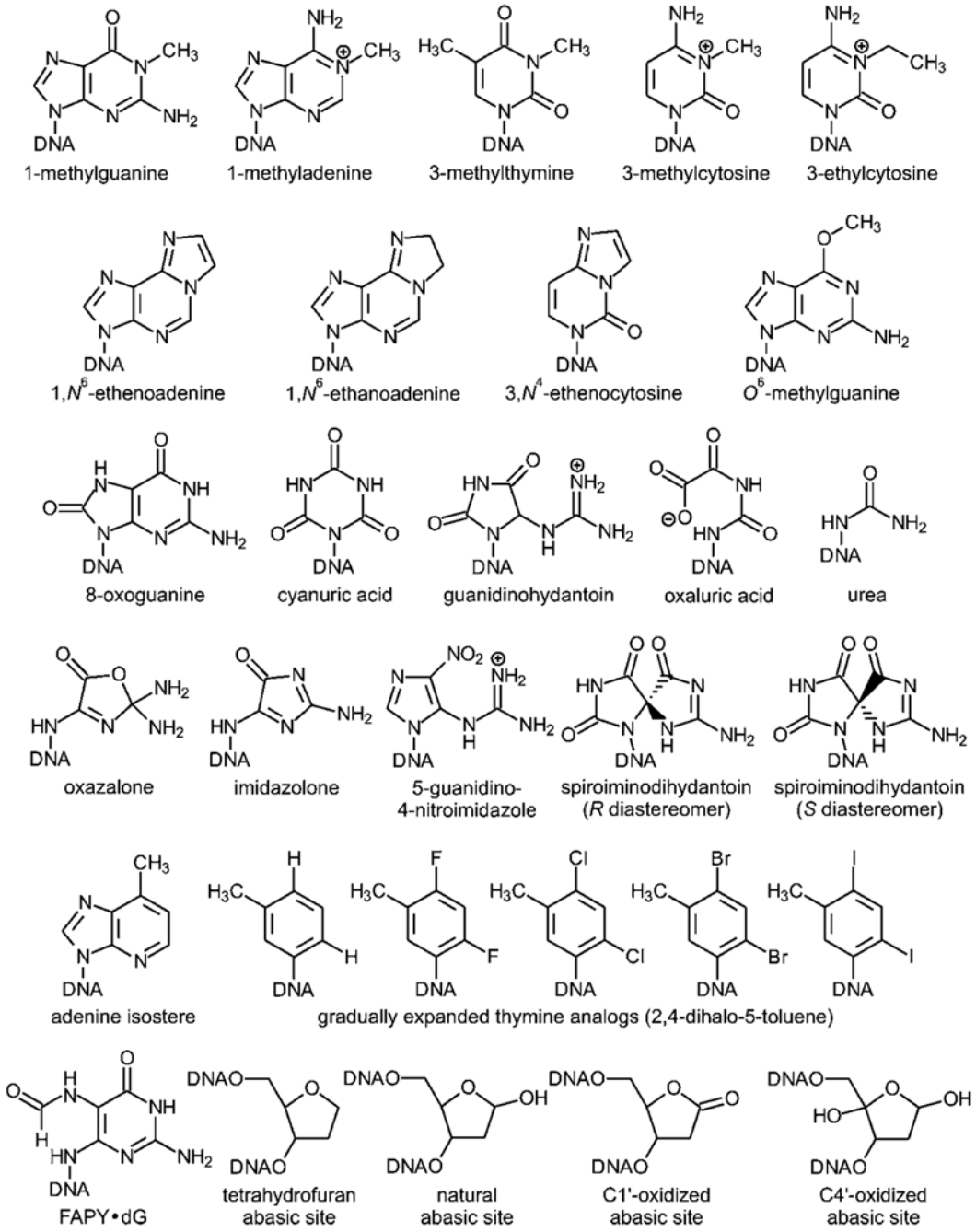

Figure 3.
Lesions analyzed by the REAP and CRAB assays.