The roles of transcription and genotoxins underlying p53 mutagenesis in vivo

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Transcription drives supercoiling which forms and stabilizes single-stranded (ss) DNA secondary structures with loops exposing G and C bases that are intrinsically mutable and vulnerable to non-enzymatic hydrolytic reactions. Since many studies in prokaryotes have shown direct correlations between the frequencies of transcription and mutation, we conducted in silico analyses using the computer program, mfg, which simulates transcription and predicts the location of known mutable bases in loops of high-stability secondary structures. Mfg analyses of the p53 tumor suppressor gene predicted the location of mutable bases and mutation frequencies correlated with the extent to which these mutable bases were exposed in secondary structures. In vitro analyses have now confirmed that the 12 most mutable bases in p53 are in fact located in predicted ssDNA loops of these structures. Data show that genotoxins have two independent effects on mutagenesis and the incidence of cancer: Firstly, they activate p53 transcription, which increases the number of exposed mutable bases and also increases mutation frequency. Secondly, genotoxins increase the frequency of G-to-T transversions resulting in a decrease in G-to-A and C mutations. This precise compensatory shift in the ‘fate’ of G mutations has no impact on mutation frequency. Moreover, it is consistent with our proposed mechanism of mutagenesis in which the frequency of G exposure in ssDNA via transcription is rate limiting for mutation frequency in vivo.

Introduction

More than half of all cancers are associated with mutations in the p53 gene. Since the p53 protein coordinates complex mechanisms of DNA repair and regulates cell growth, these relationships are disrupted when the p53 gene is inactivated, allowing for the selection of cells with higher rates of division that frequently lead to cancer. Transcription of p53 is very low in normal tissue (1), and genotoxic agents induce p53 expression (2–5). Genotoxic activity also forms oxiradicals resulting in base damage to G, which has a high oxidation potential (6,7). Earlier work with Escherichia coli (reviewed in refs 8,9) has shown that stress-related mutations are frequently located in ssDNA loops of predicted ssDNA secondary structures. Earlier work had shown correlations between starvation-induced transcription and mutation frequency (31), and the predictive value of the mfg program has since been demonstrated by showing correlations between: (i) transcription and reversion frequency in E.coli (30), (ii) reversion frequency and promoter strength (36,37), (iii) SLS stability (ΔG) and mutation rate (34), (iv) protein sequence diversity and increased levels of transcription (38) and (v) base exposure and mutation frequency in p53 (10) and, in the present study, in vitro analyses have confirmed the mfg-predicted locations of p53 hypermutable bases in loops of secondary structures.

Materials and methods

Mutation analysis

The data in Table II, Supplementary Tables 1 and 2 and Figures 1 and 2 (available at Carcinogenesis Online) pertaining to the p53 gene were obtained from the IARC TP53 database version R14 (39). This database is maintained by the International Agency for Research on Cancer. The somatic mutation dataset which contains 26 597 mutations from 2179 references was downloaded into Excel, sorted and analyzed. The data were used to examine the fate of G and C mutations in different categories of cancers and were derived by including or excluding data from original column headings as follows: the twelve most hypermutable bases from codons 175, 245, 248, 249, 273 and 282 were selected. Only ‘missense’, ‘nonsense’ or ‘silent’ mutations were included. Tumor types included were ‘primary’ and ‘NA’ (not assigned) and excluded were ‘metastasis’, ‘recurrent’ and ‘secondary’. Cancers were sorted by organ affected, and bases were sorted by fate of mutations (i.e. G-to-T, C-to-A).

Data in Table I pertaining to VH genes were obtained from the dataset in (40), Supplementary Table 1. This dataset contains 28 307 mutations in different VH genes and referenced sequences. We examined the mutations in three genes: VH5 (GenBank accession numbers X92278 and M99684), VH94 (GenBank accession number L10094) and VH186.2 (GenBank accession number L10088). The data were derived by sorting columns as follows: data under column headings: ‘Region’, CDR1 and CDR2 or FW1, FW2 and FW3 were sorted independently. For the column ‘NCBI Accession #_Vgene’, only X92278_IGHV-34-01, M99684_IGHV4-59-01, L10088_IGHV4-59-01 and L10094_IGHV3-91-01 were analyzed. In column ‘R-or-S’, R refers to missense mutations, S refers to silent mutations, and the mutations were sorted accordingly. The ‘Exchange’ column allows for sorting by fate of mutations (i.e. G-to-T, C-to-A).

The mfg program

This program is described (Figure 1) and directions for downloading and using mfg are freely available online: http://www.dbs.umt.edu/research_labs/wrightlab/upload/mfg.html. Mfg is an open source desktop application that runs on Windows, Linux and Mac operating systems.

Construction of p53-containing plasmids for generation of double-stranded DNA for SI endonuclease cleavage assays

A 1.6 kb fragment from a clone containing the entire p53 gene (Spectra Genetics, Pittsburgh, PA) was amplified by polymerase chain reaction using specific primers P5 (‘-ACCGCGTCCGCGCC’-3’) and P3 (3ex9

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Table I. Frequency and fates of G and C intrinsic instability mutations in ssDNA

<table>
<thead>
<tr>
<th>Category of cancer</th>
<th>G-to-A, intrinsic</th>
<th>G-to-T</th>
<th>G-to-C</th>
<th>C-to-T</th>
<th>C-to-A</th>
<th>C-to-G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Mutations (all)</td>
<td>2181 (74.0%)</td>
<td>664 (22.5%)</td>
<td>102 (3.5%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liverb</td>
<td>40 (12.6%)</td>
<td>272 (85.8%)</td>
<td>5 (1.6%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lungc</td>
<td>121 (33.9%)</td>
<td>206 (57.7%)</td>
<td>30 (8.4%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Head and neck</td>
<td>45 (60.0%)</td>
<td>27 (36.0%)</td>
<td>3 (4.0%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bladder</td>
<td>112 (79.4%)</td>
<td>18 (12.8%)</td>
<td>11 (7.8%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pancreas</td>
<td>43 (81.1%)</td>
<td>7 (13.2%)</td>
<td>3 (5.7%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prostate</td>
<td>20 (83.3%)</td>
<td>2 (8.3%)</td>
<td>3 (8.3%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast</td>
<td>353 (86.1%)</td>
<td>40 (9.8%)</td>
<td>17 (4.1%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Esophagus</td>
<td>231 (85.9%)</td>
<td>31 (11.5%)</td>
<td>7 (2.6%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>245 (91.8%)</td>
<td>16 (6.0%)</td>
<td>6 (2.2%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stomach</td>
<td>159 (90.3%)</td>
<td>13 (7.4%)</td>
<td>4 (2.3%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rectum</td>
<td>173 (92.5%)</td>
<td>8 (4.3%)</td>
<td>6 (3.2%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>221 (94.0%)</td>
<td>10 (4.3%)</td>
<td>4 (1.7%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colorectum</td>
<td>418 (95.9%)</td>
<td>14 (3.2%)</td>
<td>4 (0.9%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Bold type depicts tissues most prone to genotoxic stressors.

*About 5000 purine bases turn over each day due to hydrolytic depurination, with G being replaced by A because of its size (7, 22).

*In p53, hypermutable Cs are methylated and mutated directly to T.

*Silent mutations are presumed to be underreported in the IARC TP53 database because they do not result in cancer.

*Data were obtained from the VH5, VH94 and VH186.2 gene segments (see Materials and methods).

*Msh2+/− and Msh2+/+ values are similar.

Table II. Comparing the frequency and fates of p53 mutations leading to cancer in the most hypermutable G bases in 13 categories of cancer

<table>
<thead>
<tr>
<th>Category of cancer</th>
<th>Number of mutations and % of total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
</tbody>
</table>

5′-CTGAGGTTAATATCCCTCC-3′ (Integrated DNA Technologies, Coralville, IA). This segment includes the latter half of exon 5 through the end of exon 9 and was cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA) in both orientations and named 1.6 + TOPO and 1.6 – TOPO, respectively. Exon 5 alone was subcloned similarly using primers Ex5pSp5Us (5′-GCTGTTGGGTGATTC-3′) and 3′ p5ex5i (5′-GCTAGACGAT- CAGTG-3′) and named exSTOPO. Exons 5, 6, and 7 were subcloned together using primers P5 and 3′ p5ex5i (5′-TGTGGAGGTGTCAGG-3′) and named Ex5,6,7TOPO. An additional 1.6 kb fragment with the complete exon 5 segment at the 3′ end was cloned using primers 5′ p5ex5e (5′-AGCCGTCGATGCATC-3′) and 3′ p5ex5i and named 1.6 + exSTOPO. Plasmid midpreps (Promega, Madison, WI) were used to purify double-stranded DNA for cleavage assays.

SI endonuclease analysis for loop structures

A digest of 10–30 µg of double-stranded plasmid DNA was performed with 10U or 20U of S1 endonuclease (Promega) for 1 h at 37°C. DNA was then purified with a Wizard PCR clean-up system (Promega). Eluate was digested with XhoI (NEB, Ipswich, MA) for 2 h at 37°C and heat inactivated for 20 min at 65°C. Non-radioactive deoxyctydine triphosphate/deoxythymidine triphosphate/deoxyguanosine triphosphate was added together with 10 µCi of dATP and 0.5 µCi of dGTP (6000 Ci/mmol; MP Biomedical, Solon, OH) and 5U Klenowexo (NEB) to fill in and label the 5′ overhang. This procedure labels only the transcribed or non-transcribed strand of the fragment, depending on the construct. After incubation for 1 h at 37°C, the enzyme was heat inactivated for 20 min at 75°C. A 1.6 kb fragment was generated by Spel digestion, separated by agarose gel electrophoresis and purified by gel extraction columns (Omega Bio-Tek, Norcross, GA). Products were separated on a urea containing 7% polyacrylamide gel with an appropriate sequencing ladder. The primer XhoIM-S1 and the enzyme were heat inactivated for 20 min at 75°C. The control omitting S1 digestion was treated in parallel. Mung bean endonuclease (NEB) was used for 1 h at 30°C according to manufacturer’s instructions with similar results (data not shown). Sequence ladders (Sequense 2.0; USB/Affymetrix, SantaClara, CA) were generated using the same template and primers, labeled with [32P] α-ATP. Sequencing ladders are complementary to the fragments. Each reaction result was confirmed by repeating the procedure at least three times using at least two pools of DNA.

Results

Using mfg to predict the location of hypermutable bases in loops of secondary structures

Figure 1A shows an mfg computer output for a given sequence. The sequence analyzed was folded in 40 nt segments or ‘windows’, and the C at nt 233 was unpaired in 77% (% Unp) of its total folds during simulated transcription. Mfg interfaces with the mfold program which is used by mfg for folding successive single-stranded segments of a specified length for a given sequence. A window size (number of nucleotides) is chosen, and for each successive base in the sequence analyzed, mfg reports the stability (ΔG) of the most stable secondary structure (among many) in which that base is unpaired and also reports the percent of total folds in which it is unpaired. The mutability index (MI) of each unpaired base is the product of these two variables. Mfg simulates transcription in vivo by showing progressive ‘snap shot’ views of the most stable 40 nt SLSs in which each base is unpaired, similar to RNA polymerase progression along the template strand during transcription. The window size chosen relates to the rate of transcription and the amount of ssDNA folded. These simulations involve a running competition for shared nucleotides between successive interconverting secondary structures of different stabilities. Patterns emerge of secondary structures that predominate by forming repeatedly. In general, the highest stability SLSs form most frequently with those of lower stability.

In Figure 1B, transcription and associated supercoiling are modeled using a 40 nt window generating an SLS exposing a hypermutable C in exon 8 of p53. This structure (named SLS 5.0 for its ΔG) is

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Fig. 1. (A) An example of *mfg* computer output. The C nucleotide (red) at *mfg* nt 233 was selected and the most stable SLS (ΔG, −5.0) in which that base is unpaired is shown. The output also provides the window size, percent unpaired, ΔG and mutability index. (B) Transcription of the *p53* gene, resulting in negative supercoiling in the wake of the transcription complex. The SLS formed (with a ΔG of −5.0) contains hypermutable codon 282. (C) Correlation between mutation frequencies of the twelve most hypermutable bases in *p53* (39) and the *mfg*-predicted percent unpaired for each base, using a window size of 43 nts. The hypermutable base of codon 282 is arrowed. Excel was used to calculate R² values, which are statistically significant when P ≤ 0.05. (D) Activation of the *p53* promoter as measured by Chloramphenicol AcetylTransferase reporter activity as benzo[a]pyrene (B[a]P) concentration increases (4). (E) Induction of the *p53* promoter as measured by fold increase in luciferase reporter activity with increasing concentrations of the genotoxin daunomycin (2). (F) Increase in *p53* messenger RNA over time as a function of increased exposure time to genotoxin mitomycin C (5). For (C–F) n values are 13, 4, 4 and 3, respectively.
presumably formed in negative supercoils in the wake of the transcription complex and is the most stable structure in which this hypermutable C in p53 codon 282 is exposed. The mutable base is arrowed in Figure 1C which shows the correlation between mutable base exposure during transcription and the number of mutations for the 12 most mutable bases in the current IARC TP53 somatic human cancer database (39). Separate analyses determined the optimal window size for each hypermutable base (supplementary Figure 1A is available at Carcinogenesis Online), which averaged about 43 nt (10). An nfg analysis of exons 5–9 also shows that exons 5, 7, and 8 (containing the five most hypermutable p53 codons) form higher stability structures than those in exons 6 and 9 which have no hypermutable codons (supplementary Figure 1B is available at Carcinogenesis Online).

Transcription induction of the p53 gene in response to genotoxins has previously been reported (2–5). Figure 1D and E demonstrate induction of p53 with increasing levels of genotoxin, and Figure 1F shows messenger RNA levels increasing over time as cells are exposed to genotoxin, indicating the activation of transcription. There is a strong correlation between mutation and transcription frequency in both prokaryotes (Figure 2A and B) and eukaryotes, for example, in the pre-B cell line 18–81 (Figure 2C) and in the VHB1-8 antibody gene (Figure 2D) using different strength promoters in mice (29). Thus, in living systems, a correlation exists between transcription and mutation, which underlies the fundamental mechanism of mutagenesis essential to evolution. These correlations therefore represent a newly recognized key contribution to the ‘Unity in Biochemistry’, which demonstrates that metabolic mechanisms and molecules have been preserved essentially intact through billions of years of Darwinian evolution (41,42).

The G and C nucleotides in ssDNA are thermodynamically unstable and intrinsically mutable

The overall thermodynamic characteristics of nucleotides in ssDNA underlying the majority of background or ‘endogenous’ mutations are shown in Table 1A. In vivo, the ssDNA in which unpaired bases are exposed and mutable is presumably due to maintenance level metabolic activities such as transcription and replication. The hydrolytic deamination of 5-methyl C and C occur ~300 times/day/human cell, which is more than sufficient to explain the observed endogenous mutation frequency (43,44). About 5000 purine bases turn over each day due to hydrolytic depurination, with G being replaced by A at those apurinic sites because of its similar size (20). In the p53 gene, the relative mutation frequencies of Cs and Gs in missense mutations (Table 1B) and of germ line light chain antibody genes (Table 1E) are presumably also due to base exposure during transcription and intrinsic chemical instability in ssDNA. In silent mutations of p53 (Table 1C) and framework regions of antibody VH genes (Table 1D), G-to-A and C-to-T transitions also predominate. Perhaps, the most compelling evidence for a common mechanism of mutability in unpaired bases is seen in silent mutations, where the outcome of a mutation does not alter the encoded amino acid and is not selected, reflecting only intrinsic base instabilities (Table 1C and D).

Analyses of G and C mutations in the current p53 mutation database

The frequency and three possible fates of 2947 missense mutations of G-to-A, T and C in 13 different types or categories of cancer in the eight most mutable codons are summarized in Table II. Separate analyses determined the optimal window size for each hypermutable base (supplementary Figure 1A is available at Carcinogenesis Online), which averaged about 43 nt (10). An nfg analysis of exons 5–9 also shows that exons 5, 7, and 8 (containing the five most hypermutable p53 codons) form higher stability structures than those in exons 6 and 9 which have no hypermutable codons (supplementary Figure 1B is available at Carcinogenesis Online).

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**Fig. 3.** Examination of the fate of mutable Gs in p53 in all cancers compared with lung cancer. (A) The relative frequencies of p53 G-to-T mutations by tissue type. (B) The fate of G mutations in all cancers in which a majority of mutations (74.0%) are G-to-A (intrinsic). (C) The fate of G mutations in lung cancers in which a majority (57.7%) are oxyradical-induced G-to-T transversions. Mutation data are derived from missense mutations in the eight most mutable codons in the IARC TP53 database. Data for B and C are taken from Table II. (D) In all (pooled) cancers, the relative rate of G-to-A intrinsic mutations occurring in a specific G base at low levels of transcription is depicted by a 33 nt SLS and (E) in lung cancers at high levels of transcription (depicted by a 45 nt structure containing the same hypermutable G) with the approximate relative rates of G-to-A intrinsic mutations (rate of ~1) and genotoxins-oxidized G-to-T transversions (rate of ~3).
somatic hypermutation (Figure 2C and D). However, in that system, the predominant transcription-driven mutation is C-to-U due to Activation induced cytidine deaminase (AID) activation (see Discussion). As indicated in Table II (detailed in Supplementary Table 2 available at Carcinogenesis Online), the number of G-to-T transversions in liver and lung cancers is exceptionally high compared with all other cancer categories, consistent with the direct exposure of these tissues to genotoxins, which increase the rate of transcription. Toxins would be expected to accumulate preferentially in the liver, as a detoxifying organ, and in lungs, which are exposed to polluted aerosols. The distribution of G-to-T transversions in various categories of cancer is shown in Figure 3A. The dual role of genotoxins in mutagenesis is complex since genotoxins both activate transcription (exposing mutable Gs in ssDNA) and cause base damage (G-to-T transversions) primarily in lung and liver tissues (45). Figure 3B and C documents the effects of G availability and genotoxins on mutation frequency in all categories of cancer versus lung cancer. In all categories of cancer (Figure 3B), low rates of transcription and ssDNA availability are the primary sources of intrinsically mutable G-to-A mutations, similar to ‘background’ mutations (Table I), whereas at higher (genotoxin-induced) levels of transcription (Figure 3C), many Gs are oxidized to Ts, thus diverting the usual fate of G mutations away from A and to T. Since G availability via transcription is rate limiting, these compensatory shifts in the fate of G mutations have no effect on the incidence of cancer. This is also the case in Figure 3C, i.e., the incidence of cancer is the same as the number of available mutable Gs. These data indicate that the rate of availability of unpaired mutable Gs in ssDNA during transcription is rate limiting for and determines the frequency of G mutations leading to the inactivation of p53. Dynamic models help to clarify these relationships. For example, in Figure 3D in all categories of cancer, at low levels of transcription, the G is depicted to be in a single-stranded region of a 33 nt SLS of low stability (SLS 5.1). In lung cancers (Figure 3E), at higher levels of genotoxin-induced transcription (2–5), the same G is shown to be exposed in a more stable SLS at a window size of 43 nt. As discussed above, genotoxins activate p53 transcription, which increases base exposure (Figure 1C) and mutation frequency (Figure 2). Although the rate of transcription and mutable G availability determine mutation frequency, other variables such as genotoxins can affect the distribution or relative ‘fates’ of those G mutations to A, T and C as modelled in Figure 3E. However, the total number of cancers is not affected by these compensatory changes in mutation fate and is only determined by the rate of availability of intrinsically mutable Gs (Figure 3B and C). The most important variables that determine the frequency at which a mutable G is available in vivo are: (i) the frequency of transcription and unpaired base availability in ssDNA, (ii) the inherent instability and mutability of the unpaired G or C base and (iii) the extent of base exposure during transcription (Figure 1C). The type or fate of a G mutation is determined by oxynradical-induced mutations in p53 and in somatic hypermutation; a parallel may be seen in enzyme-induced AID mutations (see Discussion).

In vitro confirmation of the predicted location of loops in SLSs containing p53 codons 245 and 248

To determine whether loops in secondary structures predicted by mfg to form could be verified experimentally, we examined supercoiled plasmid DNA, which contained relevant p53 exons that had been digested with S1 endonuclease. We hypothesized that, while static, this method would provide a model to detect ssDNA exposure in secondary structure. Figure 4A shows the mfg analysis of nts 120–165 in the non-transcribed strand of exon 7, which contains hypermutable codons 245 and 248. The first three columns show the number of mutations reported in the p53 mutation database for those bases, the codons of interest and the mfg nucleotide number and the fourth column shows the stabilities (AG) of those structures. The four hypermutable bases are circled in column 6 and in SLS 9.3, SLS 12.8 and SLS 11.1, which are color coded with their location in Figure 4B and C. The location of codons 245 and 248 are bracketed black in Figure 4B and C. (see figure legends for further details). Nine S1 endonuclease cleavage sites are arrowed red in Figure 4A–D. Note the color coordination between each secondary structure and its predicted repetition (e.g. SLS 12.8 is seen 21 times in column 4, Figure 4A). The four hypermutable bases in codons 245 and 248 are unpaired and mutable (circled red) except for one G in codon 245 (SLS 12.8). Cleavage sites are immediately next to the hypermutable Gs in codon 245 in two structures (SLS 9.3 and SLS 11.1) and two cleavage sites occur in the same loop of SLS 12.8 and SLS 11.1. The two most mutable of the four hypermutable bases in codons 245 and 248 are exposed in all three structures, and the location of all cleavage sites observed experimentally (Figure 4D) correlates with their positions in loops of the relevant mfg-predicted secondary structures (Figure 4A–C). For analysis of codons 245 and 248, the structures depicted in Figure 4C represent 65% of those listed in the mfg analysis. To examine codon 175 and to verify the assumption that secondary structures formed in the two strands of a codon are similar, both strands of codon 175 were analyzed (Supplementary Figure 3A and B is available at Carcinogenesis Online). Codons 273 and 282 of exon 8 were also analyzed in the non-transcribed strand (supplementary Figure 3C and Table 1C are available at Carcinogenesis Online). Exon 8 has lower stability SLSs than exons 5 and 7; thus, the most stable SLSs observed were SLS 5.0 and 4.9 (supplementary Figure 3C is available at Carcinogenesis Online). Gels of S1 cleavage products (data not shown) revealed many faint bands, consistent with the formation of several relatively low stability structures formed from ssDNA.

In summary, separating the variables that determine the frequency of a mutation from those that determine its fate clarifies the mechanism of mutagenesis. The availability of mutable Gs in ssDNA via transcription is apparently rate limiting for, and determines the frequency of mutation, whereas the increase in oxynradical-induced base damage (G-to-T transversions), per se, has no effect on the incidence of cancer (Figure 3B and C). Given the proposed model of mutagenesis in p53, oxynradical-associated increases in the rate of transcription play a critical role in mutation frequency, and any method of downregulating the rate of p53 transcription to baseline levels (1) under stressful conditions may lower the incidence of liver and lung cancers.

Over the past 30 years, many reports on mutable bases in both prokaryotes and eukaryotes have concluded that such bases are unpaired in ssDNA loops of secondary structures, that exposed bases are chemically unstable and intrinsically mutable and that there is a direct correlation between mutation and transcription frequency. It is also known that a high mutation potential and that exposure to oxynradicals frequently results in G-to-T transversions. The proposed mechanism of mutagenesis is also supported by additional lines of evidence. In vitro analyses and structure profiling of p53 have confirmed, for the first time, that hypermutable bases in p53 are indeed located in predicted loops of high-stability secondary structures. Moreover, all 22 S1 endonuclease cleavage sites are located in loops of predicted structures, consistent with the observation that the extent of base exposure in p53 correlates directly with mutation frequency during simulated transcription (Figure 1C). Chemical analyses and silent mutations (that are not selected) have also shown that C and G nucleotides are chemically unstable and intrinsically mutable. These observations indicate that the frequency of transcription, or more specifically, the frequency of unpaired Gs and Cs in ssDNA during transcription, is rate limiting for mutation frequency in vivo.

Discussion

This work addresses a longstanding and serious problem, namely, the relevance of in vitro data to the dynamics of metabolism under steady-state conditions in vivo. Differences between metabolism in vitro versus in vivo have been the concern of a number of investigators in the past (32,33,46–51), who point out that substrates are typically used at saturating levels in vitro and enzymes at low rate-limiting concentrations in order to measure the rate of a reaction. In vivo, however (except for a few ‘pacemakers’ such as glycogen phosphorylase), enzyme concentrations under steady-state conditions usually far exceed substrate concentrations and substrate availability is
frequently more rate limiting than enzyme concentration in the intact cell. The work of Kascer (46) is an elegant example of experimental approaches that can reveal, under steady-state conditions in vivo, which enzymes are, and which are not rate controlling, depending upon their ‘kinetic position’ in the metabolic sequence. Thus, there is precedence for our conclusion that intrinsically mutable Gs and Cs are the rate-limiting substrates for mutagenesis in p53. Furthermore, available Gs and Cs in single-stranded loops of VH genes may also mutate because of intrinsic mutability and because ssDNA sites are available for mutation of C-to-U by AID-based diversification (52).

Dynamic in vivo models of mutagenesis (Figure 3D and E) show the origin and fate of transcription driven unpaired intrinsically mutable bases exposed in loops of secondary structures. The availability of these bases via transcription is apparently the rate-limiting variable that determines mutation frequency, which is also the case for mutagenesis in somatic hypermutation. Thus, the model for background mutations in p53 (Figure 3D) shows G-to-A as the intrinsically mutable rate-limiting base, many of which are usurped by genotoxins which convert them to T. In somatic hypermutation, antigen challenge increases transcription, ssDNA and mutable C availability. In this

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**Fig. 4.** S1 cleavage analysis of exon 7 of p53. Plasmid DNA containing the non-transcribed strand of exon 7 and hypermutable codons 245 and 248 was analyzed by S1 endonuclease. (A) Selected mfg computer output (15°C, 44 nt window). Column 1 shows mutation frequencies of the hypermutable bases; column 2 identifies the hypermutable codon; column 3, the mfg assigned nt number; column 4, the color-coded stability (ΔG) of the predicted highest stability SLSs containing the mutable unpaired base; column 5 contains red arrows at S1 cleavage sites; column 6 shows the sequence of the non-transcribed strand; column 7 shows the complementary transcribed strand. (B) A segment of the p53 sequence identifying the relative locations of predicted color-coded SLSs in relation to the locations of cleavage sites (red font and arrows) and the hypermutable codons (black bracket). (C) Three mfg-predicted secondary structures containing codons 245 and 248 showing S1 cleavage sites in loops (red arrows). Hypermutable bases are circled (red). (D) Polyacrylamide gel showing S1 cleavage sites (red arrowed) next to the transcribed strand sequence ladder.
case, AID activation takes most of these Cs and mutates them to Us, initiating high frequency enzyme diversification.

In both prokaryotes and eukaryotes, correlations are seen between the frequency of ssDNA availability (via transcription) and mutation frequency (Figure 2), consistent with the conclusion that substrate availability is rate limiting in vivo for mutagenesis. Mutation frequency in p53 is determined by the level of transcription, which dictates the rate of availability of exposed intrinsically mutable Gs and Cs in loops of secondary structures, and the intrinsic mutability and exposure of the relevant unpaired base. In vitro analyses in this study substantiate the existence of predicted SL5s in p53 and confirm the location of hypermutable bases in loops of those structures. We propose that the intrinsic mutability of unpaired Gs and Cs in p53, together with the rate of transcription and availability of ssDNA, determines mutation frequency in vivo. The type or fate of a mutation can be intrinsic (primarily C-to-T and G-to-A) or altered by mutagens or by enzymes.

Supplementary material

Supplementary Tables 1–3 and Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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References


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