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The production of mutant protein by a transcription-based mechanism and *in vivo* technique for determining transcriptional mutagenesis

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Abstract

When an elongating RNA polymerase encounters DNA damage on the template strand of a transcribed gene it can either be arrested by or be transcribed through the lesion. Lesions that arrest RNA polymerases are thought to be subject to transcription-coupled repair, whereas that damage that is bypassed can cause miscoding, resulting in mutations in the transcript (transcriptional mutagenesis). We have developed a technique using a plasmid-based luciferase reporter assay to determine the extent to which a particular type of DNA base modification is capable of causing transcriptional mutagenesis *in vivo*. The system uses *Escherichia coli* strains with different DNA repair backgrounds and is designed to detect phenotypic changes caused by transcriptional mutagenesis under nongrowth conditions. In addition, this method is capable of indicating the extent to which a particular DNA repair enzyme (or pathway) suppresses the occurrence of transcriptional mutagenesis. Thus, this technique provides a tool with which the effects of various genes on non-replication-dependent pathways resulting in the generation of mutant proteins can be gauged.

Introduction

Current concepts of the cellular pathways that result in the generation of mutant proteins are DNA replication-centric (1, 2). Numerous models of DNA damage-induced molecular mutagenesis mechanisms include an encounter between DNA polymerase and a damaged (modified) base with altered coding properties which results in the insertion of an inappropriate nucleotide opposite to the lesion and a subsequent change in the DNA base coding sequence. If such an event occurs in a structural gene, the result could be a codon change which is reflected in the sequence of the RNA transcript and a corresponding alteration of the amino acid sequence of the translated protein which may or may not change its function in the cell. A plethora of information exists concerning which types of DNA lesions have the ability to block or be bypassed by DNA polymerases *in vitro* and whether or not the same events occur *in vivo* as revealed by cellular toxicity or increases in mutation rates. In contrast to the substantial body of information available on the *in vitro* and *in vivo* effects of DNA damage on replication-associated events, very little is known concerning encounters between DNA lesions and the transcription machinery. The

majority of studies carried out to date have investigated the effects of relatively bulky or distortive DNA damage on its ability to cause permanent arrest of an RNA polymerase elongation complex on the template strand of a transcribed gene (35). Permanent arrest of RNA polymerase at the site of a DNA lesion is thought to serve as a signal for the initiation of transcription-coupled repair (TCR) which results in the recruitment of the DNA excision repair machinery to the site of damage (4). In contrast, several recent studies have shown that certain nondistortive DNA lesions, such as uracil, dihydrouracil, 8-oxoguanine, *O*-6-methylguanine, and abasic sites, do not arrest RNA polymerases *in vitro* (implying that this damage may not be subject to poly-merase arrest-mediated TCR) and cause base misin-sertions at the lesion site resulting in a population of mutant transcripts (6 10). If such a situation occurs in cells, the production of mutant proteins by a transcription-based mechanism could cause a change in cellular phenotype, even under nongrowth (nonreplication) conditions. This scenario has been used as one explanation for adaptive or directed mutation in nutrient-deprived bacterial cultures in which mutations appear rapidly and are nearly always of the type (revertants) that restore growth, resulting in a switch from a nondividing to dividing cellular state. In this article we describe a luciferase reporter assay that is designed to reflect a change in phenotype caused by transcriptional mutagenesis in nondividing *Escherichia coli* cells. The system takes advantage of the fact that C-terminal truncations of 10 or more amino acids in the firefly luciferase protein result in an inactive enzyme. Expression constructs can be de-signed to contain specifically engineered stop codons or base damage products on the template strand of the luciferase gene. Bypass and miscoding by RNA polymerase (transcriptional mutagenesis) at the site of base damage will result in conversion of a premature stop codon into one that changes the resulting transcript back into wild-type luciferase mRNA encoding active enzyme which is easily measured. The system can be used to gauge the ability of a large variety of different DNA base damage products to cause transcriptional mutagenesis. In addition, the effect of an intact or inactive DNA repair system on this process can also be directly assessed through the use of *E. coli* strains with different DNA repair backgrounds. Thus this technique offers a simple and relatively rapid as-say system to predict the mutagenic protein potential of various types of DNA damage under cellular non-growth conditions.

METHODS

Rationale

This method can be used to determine whether or not a particular type of DNA base modification can be bypassed by *E. coli* RNA polymerase *in vivo* and, if so, whether such bypass results in the production of a mutant transcript capable of changing the repertoire of expressed proteins in the cell. An additional feature of this system is the ability to carry out such transcriptional mutagenesis experiments in *E. coli* strains with different DNA repair backgrounds to determine the effects such repair proteins/pathways have on this event. In the example provided here, uracil is the base damage product and transcriptional mutagenesis assays are carried out in *E. coli* strains that are proficient

(*ung*+) or deficient (*ung*-) in uracilDNA glycosylase, which initiates the repair of uracil via the base excision repair (BER) pathway. Uracil is a commonly occurring base lesion in DNA which results from the spontaneous deamination of cytosine and can also be produced in cells exposed to certain chemicals or ionizing radiation. It has been previously shown that uracil, if left unrepaired, is mutagenic at the level of replication (11). Although the example described here specifies uracil as the DNA lesion to be tested for transcriptional mutagenesis (12), the method is generally applicable to any type of DNA damage that can be introduced site-specifically into an oligonucleotide. Specialized Materials To determine the effect of uracil lesions on the transcriptional/translational machinery in *E. coli*, the following vectors and bacterial strains are required.

1. pBest-luc, an *E. coli* tac luciferase expression plasmid (Promega Corp., Madison, WI).
2. Synthetic, modified template strand deoxyoligonucleotides(45-mers): 59-CGATTCCAATTCAGCGGG-GGCCACCTGATATCCTTXGTATTTAAT-39,X5 T (T-45mer), A (A-45-mer), or uracil, U (U-45-mer) for Luc-WT, Luc-STOP, and Luc-U constructs, respectively), and complementary (nontemplate) strand oligonucleotides (41-mers) 39-TAAGGTTAAGTCGCCCCCGGTG-GACTATAGGAAYCATAAAT-9,Y5 A for Luc-WT or for Luc-STOP and Luc-U constructs. Annealing of the template and nontemplate strand oligonucleotides produces a duplex DNA segment with *Pac*I*Cla*I ends (see below). To assess the effect of DNA repair systems on the potential for uracil to cause transcriptional mutagenesis, *E. coli ung*+ (BW313) and *ung*- (BW312) cells were obtained Dr. Bernard Weiss.

pBest-luc Vector modification

Strategy

The firefly luciferase reporter system allows the detection of as little as 1 3 10 220 mol of luciferase about two orders of magnitude more sensitive than the chloramphenicol acetyltransferase (CAT) system) and is ideal for measurements of bacterial protein translation from mRNA expressed under nongrowth conditions (13). In addition, the removal of the C-terminal 12 amino acids of luciferase results in a loss of 99.6% of its activity (14). We have exploited this property to deter-mine transcriptional mutagenesis in *E. coli* under non-growth conditions. A reporter assay is used to measure the levels of active luciferase generated from expression constructs derived from the pBEST-luc plasmid containing modifications in luciferase codon 445 which normally encodes lysine (K445). The pBEST-luc plasmid (4.5 kb) is restriction cut at unique sites with *Cla*I (1373) and *Pac*I (1329) to replace the 45-bp fragment with a synthetic, modified 45-bp segment containing *Pac*I*Cla*I ends with the template and nontemplate strand sequences indicated above. The *Cla*I*Pac*I duplex fragment is generated by annealing the appropriate synthetic template strand with the wild-type or stop codon-containing complementary strand, phosphorylated oligonucleotides. The Luc-STOP construct in which K445 is replaced with a stop codon should result in the production of a 15-amino-acid, C-terminal-truncated, inactive luciferase protein . UracilDNA glycosylase (en-coded by the *E. coli ung* gene) initiates repair of

uracil lesions in DNA via the base excision repair pathway and is required for uracil removal (15). To determine the extent that *ung*-mediated repair of uracil in the Luc-U construct occurs under the conditions of the transcriptional mutagenesis experiments, a U/T base mismatch was created at the position of uracil in codon 445. Repair of this uracil would result in the conversion of codon 445 back to a stop codon in the template strand, resulting in the production of a truncated, inactive luciferase. Alternatively, transcription through and insertion of adenine opposite to uracil (transcriptional mutagenesis) would produce a lysine codon and restore the wild-type amino acid sequence, resulting in functional luciferase. This system provides a straightforward reporter assay to distinguish between transcriptional mutagenesis and repair of this lesion under cellular nongrowth conditions (12). In the event of transcriptional arrest at uracil, a truncated inactive luciferase protein would also be produced.

Quality Control of Damage-Containing Duplex Oligonucleotides

The nature of the synthetic duplex oligonucleotide (45-mer) to be used for replacement of the wild-type pBest-luc *Pac*I*Cla*I fragment should be verified by base-specific chemical cleavage (MaxamGilbert) DNA sequencing before use in transcriptional mutagenesis experiments. In addition, DNA damage-containing duplex oligonucleotides should be further verified by treatment with an appropriate DNA N-glycosylase or damage-specific endonuclease followed by strand scission analysis on a DNA sequencing-type gel (16). In the case of the uracil-containing oligonucleotide used in this example, the placement of uracil at the correct nucleotide position is verified by treatment of the synthetic, 39-end-labeled *Cla*I/*Pac*I fragment with purified uracilDNA glycosylase (Epicenter Technologies, Madison, WI).

Verification of Uracil in Duplex Oligonucleotide

1. 39-End-label uracil-containing, single-stranded 45-mer using terminal deoxynucleotidyltransferase and dideoxy [a-32P]ATP (Amersham, Piscataway, NJ, 3000 Ci/mmol) as previously described (17). Purify the resulting 39-end-labeled single-stranded oligonucleotide on a 20% denaturing (7 M urea) polyacrylamide gel. Following annealing with the complementary strand, purify 39-end-labeled duplex oligonucleotide on a 20% nondenaturing polyacrylamide gel as previously described (18). Resuspend uracil-containing, duplex oligonucleotide (URA-45-mer) in 10 mM HepesKOH, pH 8.0, 2 mM EDTA (HE buffer) and store at 220°C.
2. Incubate end-labeled, duplex URA-45-mer (2050 pmol) with uracilDNA glycosylase (6 units) for 30 min at 30°C in UDG buffer (30 mM HepesKOH, pH 7.5, 1 mM EDTA, 50 mM NaCl) to generate the abasic site-containing oligonucleotide, which is extracted with PCIA (phenol:chloroform:isoamyl alcohol, 29:19:1, v/v/v, equilibrated with HE buffer and 0.1% 8-hy-droxyquinoline) and is evaluated for its AP site content by cleavage with 1.0 M piperidine at 90°C for 20 min (18). Electrophorese reaction products on a 20% denaturing polyacrylamide gel, and subject to autoradiography and phosphorimager analysis.

Luciferase Expression in E. coli under Nongrowth Conditions

In the presence of 50 mM novobiocin, DNA synthesis was completely inhibited (reduced to background levels) over a 240-min period following isopropyl- b-D-1 thiogalactopyranoside (IPTG) induction of the luciferase gene (12). In contrast, RNA synthesis occurred under these conditions. When the RNA synthesis inhibitor rifampicin was added to the cells, RNA synthesis and luciferase activity were completely eliminated, indicating that the luciferase activity detected in this assay resulted from RNA synthesis-dependent translation of luciferase mRNA. Optical density measurements were also conducted to confirm that no cell growth occurred under these conditions (12).

DNA and RNA Synthesis Measurements

DNA synthesis is determined by pulse labeling (19) at 0, 20, 60, 120, and 240 min following electroporation of Luc-WT, Luc-STOP, or Luc-U into cells. Aliquots of 0.4 ml of bacterial culture are pulsed with 2 mCi of [3 H-*methyl*]thymidine (Amersham, 75.0 Ci/mmol) for 5 min. The reaction is stopped by adding an equal volume of 10% trichloroacetic acid (TCA) to cells and holding on ice for 30 min. TCA precipitates are collected on 0.45- mm Millipore (Bedford, MA) GF/A filters and rinsed with 5% TCA. The filters are dried and counted in a scintillation counter. Growth-inhibited cultures (LB-novobiocin) are compared with growing cultures (LB) (20). The amount of novobiocin used for nongrowth conditions is titrated to 50 mM, which is found to completely inhibit DNA synthesis but allow

RNA synthesis and expression of luciferase.

RNA synthesis is determined by pulse labeling as previously described with slight modification (21). As described above for the DNA synthesis measurements, following electroporation of Luc-WT, Luc-STOP, or Luc-U constructs into cells, 0.4-ml aliquots of bacterial culture are pulsed with 2 mCi of [5- 3 H]uridine (Amer-sham, 2530 Ci/mmol) for 5 min at the same time points used in the DNA synthesis experiments. Ten percent TCA is added to stop the reaction which was then held on ice for 30 min. Precipitates are collected on 0.45- mm Millipore GF/A filters and rinsed with 5% TCA. The filters are dried and counted. Growth-inhibited cultures (LB-novobiocin) are compared with rifampicin (150 mg/ml)-treated cultures and growing cultures (LB alone).

Induction of Luciferase in Nondividing ung+ or ung- E. coli Cells

Uracil is repaired by uracilDNA glycosylase (encodedby the *ung* gene) which initiates removal of uracil in DNA via the base excision repair pathway. To assess the effect of DNA repair on the potential for uracil to cause transcriptional mutagenesis, it is necessary to conduct parallel experiments with Luc-WT-, Luc-STOP-, and Luc-U-transformed cells in *E. coli ung* + and *ung* - backgrounds. Differences in the level of luciferase production in these uracil repair-proficient (*ung* +) and repair-deficient (*ung* -) strains reveal the extent to which such DNA repair systems affect lesion-driven transcriptional mutagenesis under nongrowth conditions. In the uracil repair-deficient (*ung* -) strain, in the absence

of DNA synthesis, Luc-U supported synthesis of active luciferase, indicating transcriptional mutagenesis occurs under these conditions (Table 1). As described in detail above, the Luc-WT, Luc-STOP, or Luc-U constructs are electroporated into *ung* + or *ung* - *E. coli* and subsequently incubated in novobiocin containing LB medium for 30 min. Aliquots of cells are plated onto LB Amp medium to determine transformation efficiency. Luciferase activity is determined 120 min following IPTG induction of luciferase. Normalized (adjusted) luciferase activity was determined on the basis of transformation efficiency and total luciferase activity (12).

Preparation of Cell Extracts and Luciferase Enzyme Assays

1. Harvest bacterial cells and place in luciferase lysis buffer (25 mM Tris-phosphate (pH 7.8), 2 mM DTT, 2 mM 1,2-diaminocyclohexane-*N,N,N9,N9*-tetraacetic acid, 10% glycerol, 1% Triton X-100).
2. Quick-freeze cells and lyse by freeze-thawing five times.
3. Mix 20 ml of bacterial cell extract with 100 ml of luciferase assay reagent (Promega Corp.) at room temperature.
4. Place the reaction in a luminometer (Zylux Corp., Maryville, TN, Femtomaster Model FB12); measure the luminescence level (RLU units) for 10 s.
5. Calculate transformation efficiency (number of colonies per microgram of DNA) as the number of Amp R colonies per microgram of DNA and subsequently use to normalize luciferase activity values obtained for each strain.
6. As shown in Table 1, the Luc-U construct produced an approximately 200-fold increase in luciferase in the *ung* strain compared with the *ung* + strain. This difference could not be accounted for by potential differences in the transcriptional and translational machineries between the two strains as the levels of luciferase produced from the Luc-WT construct in the *ung*1 and *ung*- cells are essentially the same. This result provides direct evidence that uracil causes transcriptional mutagenesis *in vivo* and results in a phenotypic change in nondividing cells.

CONCLUDING REMARKS

The ability of uracil and other types of DNA base damage to cause transcriptional mutagenesis will most likely depend on how rapidly they can be recognized and eliminated by the various proteins involved in the initiation of their repair. Under conditions of active transcription, uracil may be repaired rapidly and efficiently whereas other types of lesions may be reversed more slowly, even in a wild-type DNA repair background. The level to which an RNA polymerase miscoding lesion is capable of inducing transcriptional mutagenesis will most likely depend on the nature of the lesion itself, which will, in turn, dictate the repair systems available (and whether or not they are impaired in any way) as well as the extent of transcription taking place on the gene. The method described here makes it possible to determine which classes of DNA lesions cause transcriptional bypass and miscoding, resulting in a phenotypic change *in vivo*. The ability to use this technique in bacterial strains with different DNA repair back-grounds further enhances the value of

this method. Information gained from these systems can also be used to understand whether similar processes occur in eukaryotic systems and how they may be affected by the genetic background of the cell.

TABLE 1

Effect of DNA Repair Status on Transcriptional Base Substitution

	Normalized luciferase activity (RLU/106)				
Construct	ung- strain		ung+ strain		Ratio ung-/ ung+
Luc-WT	112,559	2164	124,360	8878	~ 1
Luc-STOP	326	54	417	62	~ 1
Luc-U	79,514	6166	380	183	209

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