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Phenotypic Change Caused by Transcriptional Bypass of Uracil in Nondividing Cells

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Cytosine deamination to uracil occurs frequently in cellular DNA. In vitro, RNA polymerase efficiently inserts adenine opposite to uracil, resulting in G to A base substitutions. In vivo, uracil could potentially alter transcriptional fidelity, resulting in production of mutant proteins. This study demonstrates that in nondividing *Escherichia coli* cells, a DNA template base replaced with uracil in a stop codon in the firefly luciferase gene results in conversion of inactive to active luciferase. The level of transcriptional base substitution is dependent on the capacity to repair uracil. These results provide evidence for a DNA damage–dependent, transcription-driven pathway for generating mutant proteins in nondividing cells.

Numerous studies have shown that different types of DNA damage exert deleterious effects on replication by either blocking DNA polymerase or allowing for mutagenic bypass (*1*). Cells that are unable to repair certain types of DNA damage have increased mutation rates (*2*, *3*). In contrast, much less is known concerning the interaction of DNA damage and RNA polymerases during transcription, with most studies focusing on lesions that permanently arrest RNA polymerase during elongation. An arrested RNA polymerase complex at a site of DNA damage serves as a signal for recruitment of the DNA excision repair machinery (*4*). Transcription-coupled repair allows for template strand-specific repair of a number of RNA polymerase-arresting lesions such as cyclobutane pyrimidine dimers and thymine glycol (*5*). However, a number of different DNA base modifications including uracil do not arrest RNA polymerase in vitro and miscode at the lesion site, producing mutant transcripts with high efficiency (*6*–*11*). An unexplored possibility is that if similar events occur in vivo, the resulting mutant transcripts will generate mutant proteins that could alter the cellular phenotype (*6*, *12*–*14*).

Uracil is a frequently occurring DNA base damage that results from the spontaneous or chemically induced deamination of cytosine and is mutagenic at the level of replication (*3*, *15*). To determine the effect of uracil on transcription and translation in *Escherichia coli*, we used a reporter assay to measure the levels of active firefly luciferase generated from expression constructs derived from the plasmid pBest-luc containing modifications in the luciferase lysine codon 445 (Fig. 1). A unique 45–base pair (bp) Pac I–Cla I restriction fragment flanking Lys^{445} was removed from this construct and replaced with one of three fragments that contained in the template strand

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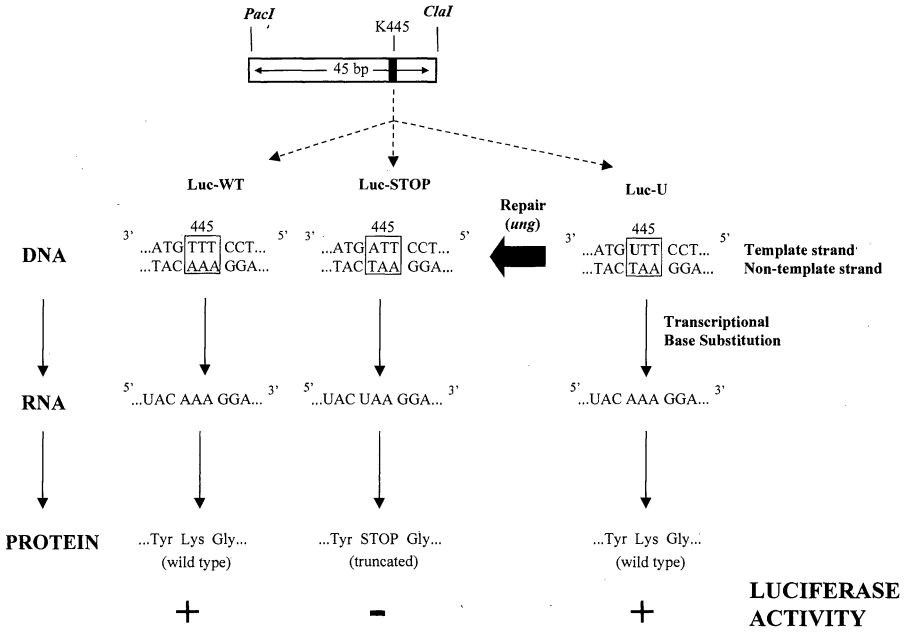

Fig. 1. Strategy for determining transcriptional base substitution in cells: luciferase gene modification. The pBest-luc luciferase reporter construct contains the firefly luciferase gene driven by the *E. coli* tac promoter and also contains the ampillicin (Amp) resistance gene (*15*). The 45-bp Cla I–Pac I fragment of pBest-luc was modified on the template strand at the first base of codon 445 to specify either lysine (3′-TTT-5′; Luc-WT), a stop codon (3′-ATT-5′; Luc-STOP), or lysine through transcriptional base substitution at uracil (3′-UTT-5′; Luc-U), resulting in production of wild-type, truncated, or transcriptional base substitution–mediated wild-type luciferase proteins, respectively. If DNA repair occurs through the *ung*-mediated BER pathway, codon 445 in the Luc-U construct would be converted to a stop codon (3′-ATT-5′), resulting in a truncated, inactive luciferase protein. The constructs were electroporated into ung^+ or ung^- (or in some cases $mutS^+$ or $mutS^-$) *E. coli* strains and subsequently incubated in novobiocin-containing LB media for 30 to 240 min. Portions of cells were also plated onto LB-Amp media to determine transformation efficiency. Luciferase activity and DNA and RNA synthesis were determined from 30 to 240 min after IPTG induction. Normalized luciferase activity was determined on the basis of transformation efficiency and total luciferase activity (*32*). K445, Lys^{445}.

either a stop codon (3′-ATT-5′, Luc-STOP construct), a uracil (U) substitution for one of the stop codon bases (3′-UTT-5′, Luc-U construct), or the wild-type (WT) codon (3′-TTT-5′, Luc-WT construct) (*16*). The Luc-U construct contains a U/T mismatch at the position of uracil to assess the potential effect of repair by uracil-DNA glycosylase (encoded by the *ung* gene) in this system (Fig. 1). The Luc-STOP construct, in which Lys^{445} is replaced with a stop codon, should result in the production of a 15–amino acid, COOH-terminal–truncated, inactive luciferase protein (*17*). The expression of these luciferase constructs was driven by the endogenous *E. coli* tac promoter in cells maintained under nongrowth conditions (Fig. 1). Using the DNA gyrase inhibitor novobiocin, which blocks DNA synthesis and cell division (*18*), we examined transcription-related events in vivo by monitoring luciferase activity in the presence of this drug. Novobiocin effectively inhibits both chromosomal and plasmid replication (*19*) and does not induce the SOS DNA repair response in *E. coli* (*20*). Under the nongrowth conditions required for these experiments, the levels of DNA and RNA synthesis in *E. coli* were determined (*21*). In the presence of 50 μM novobiocin, DNA synthesis was completely inhibited over a 240-min period after isopropyl-β-D-1-thiogalactopyranoside (IPTG) induction of the luciferase gene (Fig. 2A). In contrast, RNA synthesis occurred under these conditions (Fig. 2B). In the presence of rifampicin, RNA synthesis (Fig. 2B) 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 confirmed that no cell growth occurred (Fig. 2C) (*22*).

Expression of Luc-STOP in uracil repair-proficient (ung^+) or repair-deficient (ung^-) cells generated a truncated, inactive luciferase protein compared with that produced upon expression of Luc-WT, which generated active, wild-type luciferase in both ung^+ and ung^- strains (Fig. 3A). Uracil-DNA glycosylase initiates removal of uracil in DNA through the base excision repair (BER) pathway (*23*). In the event that *ung*-mediated repair of uracil in the Luc-U construct occurred under these conditions, a U/T base mismatch was created at the uracil position in codon 445 (Fig. 1). Repair of this uracil would convert codon 445 back to a stop codon in the template strand, resulting in production of inactive luciferase. Alternatively, transcription through and insertion of adenine opposite to uracil would convert the stop codon to a lysine codon and restore the wild-type amino acid sequence, resulting in active luciferase. This system provides a straightforward method to distinguish between transcriptional base substitution or repair of uracil under cellular nongrowth conditions. In the event of transcriptional arrest at uracil, a truncated, inactive luciferase protein would also be produced.

To assess the effect of DNA repair capacity on the potential for uracil to cause transcriptional base substitution, we carried out parallel experiments with Luc-WT–, Luc-STOP–, and Luc-U–transformed cells in *E. coli* ung^+ and ung^- strains. In the uracil repair-deficient (ung^-) strain, Luc-U supported synthesis of active luciferase, indicating that transcriptional base substitution occurs under these conditions (Fig. 3A). Comparison with the Luc-WT control revealed that Luc-U is able to restore luciferase activity to nearly wild-type levels in the absence of uracil repair. In contrast, in ung^+ cells, luciferase activities from Luc-U transformants were not above background levels, indicating that repair of uracil completely suppresses transcriptional base substitution. Because DNA gyrase is not required for BER-related DNA synthesis (*24*), uracil removal can still occur in the presence of novobiocin.

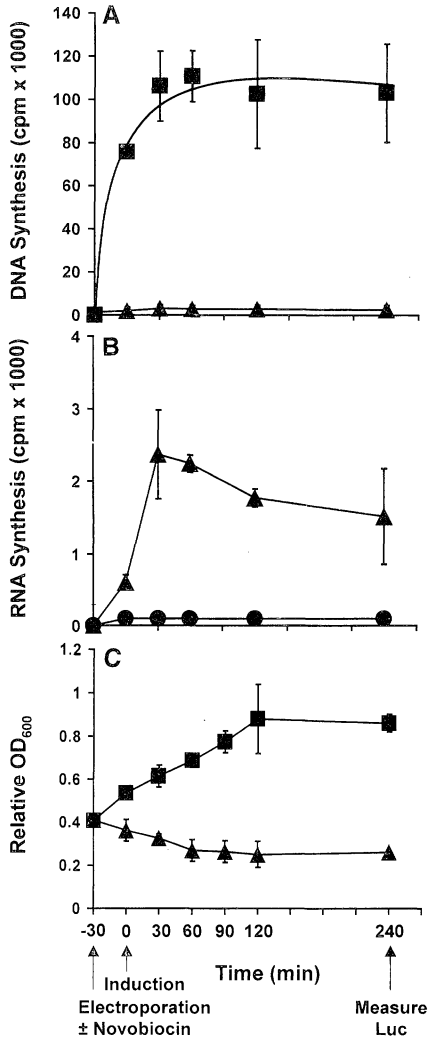

Fig. 2. DNA and RNA synthesis and cell density under growth and nongrowth conditions. (**A**) DNA synthesis in the presence or absence of novobiocin. DNA synthesis was measured by pulse labeling with [methyl-^{3}H]thymidine as described (*21*, *30*). The −30-min time point corresponds to the time immediately after electroporation. Cells were induced with IPTG at 0 min and were placed in either LB media alone (growth conditions, closed squares) or LB-novobiocin (nongrowth conditions, closed triangles). Luciferase measurements (indicated by arrow) were made at 240 min. (**B**) RNA synthesis in the presence of novobiocin or rifampicin. RNA synthesis was measured by pulse labeling with [5-^{3}H] uridine as described (*21*, *31*). The –30-min time point corresponds to the time immediately after electroporation. Cells were induced with IPTG at 0 min and were grown in LB-novobiocin (nongrowth conditions, closed triangles) or in the presence of rifampicin (150 μg/ml) (negative control, closed circles). Luciferase measurements (indicated by arrow) were made at 240 min. (**C**) Cell growth in the presence or absence of novobiocin. The level of cell growth was measured by optical density measurements at 600 nm (OD_{600}). The –30-min time point corresponds to the time immediately after electroporation. Cells were induced with IPTG at 0 min and were placed in either LB media alone (growth conditions, closed squares) or LB-novobiocin (nongrowth conditions, closed triangles). Optical density at each time point was measured after a 10-fold dilution of a fraction of the cultures. Data shown represent values obtained in ung^- cells. Similar results were obtained with an isogenic ung^+ strain. Values reported are the average of three experiments, and error bars represent one standard deviation.

Table 1. Effect of DNA repair status on transcriptional base substitution. Normalized luciferase activity for Luc-WT, Luc-STOP, and Luc-U constructs in *E. coli* uracil repair-proficient (ung^+) and repair-deficient (ung^-) strains. Values for ung^-/ung^+ ratios are rounded to nearest whole number values. Average luciferase activities (RLU/10^6) from three separate experiments are shown.

Construct	Normalized luciferase activity (RLU/10^6)		Ratio ung^-/ung^+
	Strain		
	ung^-	ung^+	
Luc-WT	112,559 ± 2,164	124,360 ± 8,878	~1
Luc-STOP	326 ± 54	417 ± 62	~1
Luc-U	79,514 ± 6,166	380 ± 183	209

It is also possible that the U/T mismatch present in the Luc-U construct could be recognized and altered by the *E. coli* MutHLS mismatch repair system, which hypothetically could act on either strand. We consider this possibility unlikely for several reasons. First, when the Luc-WT, Luc-STOP, and Luc-U constructs were tested in a uracil repair-proficient, mismatch repair-deficient (ung^+, $mutS^-$) background, luciferase activity levels were the same as those observed for an ung^+, $mutS^+$ background (Fig. 3B). This result rules out mismatch repair of the T in the U/T mispair of the Luc-U construct, which would result in production of a U/A pair and conversion to wild-type luciferase. Second, the magnitude of the effect of the loss of uracil-DNA glycosylase (ung^-) on luciferase activity levels provides direct evidence that the inability to specifically repair uracil (as opposed to a mismatch) leads to production of active luciferase (Fig. 3A). In addition, T/T (equivalent to U/T) base mispairs are poorly recognized by *E. coli* mismatch repair proteins (*25*), and MutS and MutH proteins decline to low levels under nongrowth conditions (*26*).

The Luc-U construct produced a 200-fold increase in luciferase in the ung^- strain compared with the ung^+ strain (Fig. 3A and Table 1). 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 ung^+ and ung^- cells are essentially the same (Table 1). The same relative pattern of luciferase activity was obtained for the Luc-WT and Luc-U constructs throughout a range (25 to 100 ng) of transfected DNA amounts, indicating that the level (50 ng) of DNA used in these experiments was not saturating for either the ung^+ or ung^- strains (Fig. 3C). This striking effect observed in ung^- cells indicates that in the case of uracil present on the template strands of transcribed genes, the absence of uracil-DNA glycosylase-initiated BER results in a large increase in transcriptional base substitution. These results provide insights into the role of the BER pathway in preventing transcriptional errors. Under conditions at which the repair of uracil is impaired, transcriptional base substitution at sites of uracil may be a predominant mechanism for the production of mutant proteins, especially in nondividing cells. The same situation may also exist for other types of DNA damage such as dihydrouracil, 8-oxoguanine, and O^6-methylguanine, which also cause RNA polymerase errors in vitro (*7*, *8*, *10*). Thus, the extent to which an RNA polymerase miscoding lesion is capable of inducing transcriptional base substitution will probably depend on the nature of the lesion itself, the type of repair systems available, and the level of transcription taking place on the gene.

The potential biological outcomes of transcriptional base substitution could include a number of deleterious events initiated by mutant proteins, including cell death or an alteration of cellular physiology, such as initiation of DNA replication. In this regard, it is important to point out that many DNA base damages that cause RNA polymerase misinsertions cause the same type of misinsertions for DNA polymerases during synthesis (*6*, *8*, *10*). Transcriptional base substitution–mediated events leading to the initiation of DNA replication could result in permanently "fixing" a mutation into the genome if the miscoding damage is left unrepaired when it encounters the DNA polymerase (*6*, *12*, *13*). Such a situation could produce a switch from a nongrowth to a growth state in a previously quiescent cell, resulting in a dividing population of mutants (*6*, *13*). The recent finding that β-amyloid protein transcripts from neurons in patients with Alzheimer's disease contain mutations not found at the respective DNA level when compared with healthy controls can be explained by transcriptional base substitution (*27*). In another context, a buildup of damage-

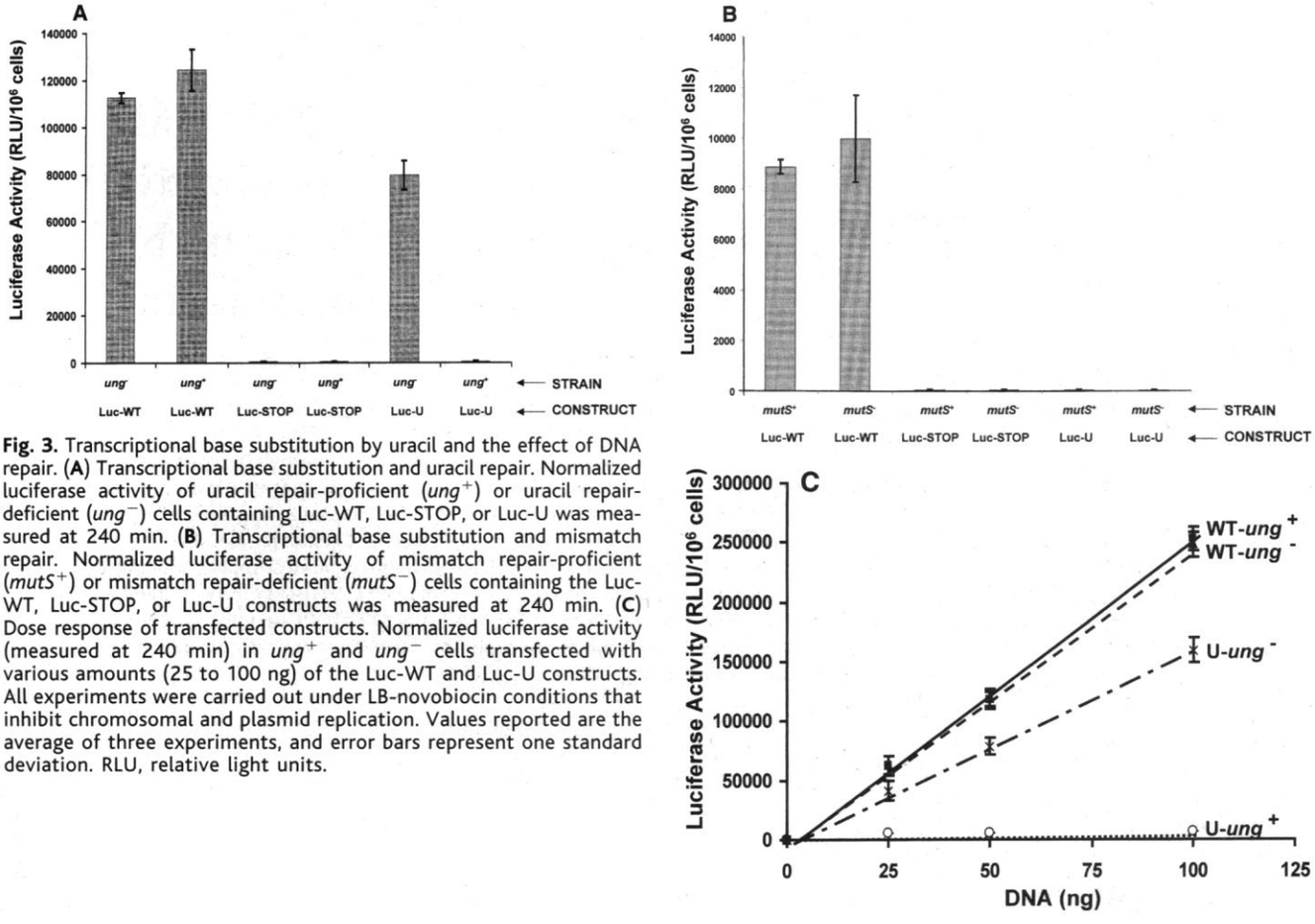

Fig. 3. Transcriptional base substitution by uracil and the effect of DNA repair. (**A**) Transcriptional base substitution and uracil repair. Normalized luciferase activity of uracil repair-proficient (ung^+) or uracil repair-deficient (ung^-) cells containing Luc-WT, Luc-STOP, or Luc-U was measured at 240 min. (**B**) Transcriptional base substitution and mismatch repair. Normalized luciferase activity of mismatch repair-proficient ($mutS^+$) or mismatch repair-deficient ($mutS^-$) cells containing the Luc-WT, Luc-STOP, or Luc-U constructs was measured at 240 min. (**C**) Dose response of transfected constructs. Normalized luciferase activity (measured at 240 min) in ung^+ and ung^- cells transfected with various amounts (25 to 100 ng) of the Luc-WT and Luc-U constructs. All experiments were carried out under LB-novobiocin conditions that inhibit chromosomal and plasmid replication. Values reported are the average of three experiments, and error bars represent one standard deviation. RLU, relative light units.

related transcriptional errors leading to mutant proteins could result in "error catastrophe" where genes necessary for cellular viability become compromised, resulting in age-related cell death (*28*). This mechanism may also provide one explanation for adaptive (or directed) mutagenesis (*29*). Future studies should be focused on determining the extent to which transcriptional base substitution contributes to the mutant protein burden of cells.

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16. pBest-luc, an *E. coli* tac-luciferase expression plasmid was obtained from Promega (Madison, WI). This plasmid was cut at unique sites with Cla I (1373) and Pac I (1329) to replace the 45-bp segment with a synthetic, modified fragment (Cla I–Pac I fragment; template strand 5′-CGATTCCAATTCAGCGGGGGCCACCTGATATCCTTXG-TATTTAAT-3′; X is T, A, or U for Luc-WT, Luc-STOP, and Luc-U constructs, respectively). The Cla I–Pac I duplex fragment was generated by annealing the appropriate synthetic template strand with the wild-type or stop codon–containing, complementary strand, phosphorylated oligonucleotides (Gibco-BRL Life Technologies, Palo Alto, CA). The placement of uracil at the correct nucleotide position was verified by treatment of the synthetic, end-labeled Cla I–Pac I fragment with purified uracil-DNA glycosylase (Epicenter Technologies) and *E. coli* endonuclease IV (gift from R. P. Cunningham), which generated the appropriate DNA cleavage product when analyzed on a DNA sequencing gel. Five hundred micrograms of purified linear vector was used in a ligation reaction with 8 μg of the Cla I–Pac I fragment containing either a wild-type, stop, or uracil codon. The resulting Luc-WT, Luc-STOP, and Luc-U constructs were expressed in *E. coli* ung^+, ung^-, $mutS^+$, and $mutS^-$ cells by electroporation of 50 ng of DNA.
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21. DNA synthesis was determined by pulse labeling as previously described (*30*). Briefly, at 0, 20, 60, 120, and 240 min after electroporation of Luc-WT, Luc-STOP, or Luc-U into cells, 0.4-ml portions of bacterial culture were pulsed with 2 μCi of [^{3}H-methyl]thymidine (Amersham; 75.0 Ci/mmol) for 5 min. The reaction was stopped by adding an equal volume of 10% trichloroacetic acid (TCA) to cells and holding on ice for 30 min. TCA precipitates were collected on 0.45-μm Millipore GF/A filters and rinsed with 5% TCA. The filters were dried and counted in a Wallac 1409 scintillation counter (Gaithersburg, MD). Growth-inhibited cultures [LB-novobiocin (50 μM)] were compared with growing cultures (LB alone) (*19*). The amount of novobiocin used for nongrowth conditions was titrated to 50 μM, which was found to completely inhibit DNA synthesis but allowed RNA synthesis and expression of luciferase. RNA synthesis was determined by pulse labeling as previously described with slight modifications (*31*). As described above for the DNA synthesis measurements, after electroporation of Luc-WT, Luc-STOP, or Luc-U constructs into cells, 0.4-ml portions of bacterial culture were pulsed with 2 μCi of [5-^{3}H]uridine (Amersham; 25 to 30 Ci/mmol) for 5 min at the same time points used in the DNA synthesis experiments. Ten percent TCA was added to stop the reaction, which was then held on ice for 30 min. Precipitates were collected on 0.45-μm Millipore GF/A filters and rinsed with 5% TCA. The filters were dried and counted. Growth-inhibited cultures [LB-novobiocin (50 μM)] were compared with rifampicin-treated cultures [LB-rifampicin (150 μg/mL)] and growing cultures (LB alone).
22. Competent *E. coli* cells were electroporated with DNA and incubated at 37°C in 1 ml of LB media in the presence or absence of novobiocin. Optical density (600 nm) measurements to determine cell growth were conducted at 0 to 240 min after IPTG induction by removing 100 μl and diluting the cells to 1 ml in LB media.
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32. After electroporation of Luc-WT, Luc-STOP, and Luc-U constructs, bacteria were incubated in LB-novobiocin (50 μM) media for 30 min and then subsequently induced with IPTG for 240 min. After the induction period, cells were harvested and placed in a luciferase lysis buffer [25 mM tris-phosphate (pH 7.8), 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol, and 1% Triton X-100] and lysed by freeze-thawing five times. The assay for luciferase activity was performed with the Luciferase Assay Kit (Promega), according to the manufacturer's instructions. Transformation efficiency (number of colonies per microgram of DNA) was calculated as the number of Amp^R colonies per microgram of DNA and was subsequently used to normalize luciferase activity values obtained for each strain used in these studies. *Escherichia coli* ung^+ (BW313) and ung^-(BW312) cells were obtained from B. Weiss. *Escherichia coli* $mutS^-$ (ES1301) was obtained from the *E. coli* stock center of Yale University (New Haven, CT). *Escherichia coli* $mutS^+$ (ES943) was obtained from E. Siegal.

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Dynamic Control of CaMKII Translocation and Localization in Hippocampal Neurons by NMDA Receptor Stimulation

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Calcium-calmodulin–dependent protein kinase II (CaMKII) is thought to increase synaptic strength by phosphorylating postsynaptic density (PSD) ion channels and signaling proteins. It is shown that *N*-methyl-D-aspartate (NMDA) receptor stimulation reversibly translocates green fluorescent protein–tagged CaMKII from an F-actin–bound to a PSD-bound state. The translocation time was controlled by the ratio of expressed β-CaMKII to α-CaMKII isoforms. Although F-actin dissociation into the cytosol required autophosphorylation of or calcium-calmodulin binding to β-CaMKII, PSD translocation required binding of calcium-calmodulin to either the α- or β-CaMKII subunits. Autophosphorylation of CaMKII indirectly prolongs its PSD localization by increasing the calmodulin-binding affinity.

CaMKII is involved in synaptic plasticity (*1*) and in the maintenance of the dendritic architecture (*2*). These different postsynaptic and dendritic functions of CaMKII make this enzyme a good model to investigate if and how signaling proteins are recruited to their sites of action. We studied the translocation of CaMKII isoforms by monitoring green fluorescent protein (GFP)–tagged CaMKII isoforms in cultured hippocampal CA1-CA3 neurons (*3*). GFP–α-CaMKII was uniformly distributed in the cell body and main processes of living neurons [Fig. 1A, (I)]. Glutamate stimulation induced a rapid enrichment of fluorescence in punctate structures along the dendritic processes and somatic cortex [Fig. 1A, (II)]