Environmental stress induces trinucleotide repeat mutagenesis in human cells

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The dynamic mutability of microsatellite repeats is implicated in the modification of gene function and disease phenotype. Studies of the enhanced instability of long trinucleotide repeats (TNRs)—the cause of multiple human diseases—have revealed a remarkable complexity of mutagenic mechanisms. Here, we show that cold, heat, hypoxic, and oxidative stresses induce mutagenesis of a long CAG repeat tract in human cells. We show that stress-response factors mediate the stress-induced mutagenesis (SIM) of CAG repeats. We show further that SIM of CAG repeats does not involve mismatch repair, nucleotide excision repair, or transcription, processes that are known to promote TNR mutagenesis in other pathways of instability. Instead, we find that these stresses stimulate DNA rereplication, increasing the proportion of cells with >4 C-value (C) DNA content. Knockdown of the replication origin-licensing factor CDT1 eliminates both stress-induced rereplication and CAG repeat mutagenesis. In addition, direct induction of rereplication in the absence of stress also increases the proportion of cells with >4C DNA content and promotes repeat mutagenesis. Thus, environmental stress triggers a unique pathway for TNR mutagenesis that likely is mediated by DNA rereplication. This pathway may impact normal cells as they encounter stresses in their environment or during development or abnormal cells as they evolve metastatic potential.

stress-induced mutagenesis | trinucleotide repeats | DNA rereplication | human cells | microsatellite repeats

Microsatellite repeats—runs of one to eight nucleotides repeated in tandem—account for 3% of the DNA in the human genome (1). These repeats are remarkably dynamic, mutating at rates several orders of magnitude higher than the rates of point mutations (2, 3). Microsatellite mutations typically change the number of repeated units in the tract, adding or removing individual units, making the mutations predictable and readily reversible, in contrast to point mutations. Growing evidence implicates microsatellite repeats as modifiers of gene function, traits, and diseases.

Changes in the lengths of microsatellite repeats in noncoding regions, for example, can modulate promoter activity, altering the level of gene expression (4–7). In addition, some pathogenic bacteria use frequent mutations of coding-region repeats to shift the reading frame, switching key phase-variation genes on or off to avoid host immune defenses (2). Finally, different lengths of coding trinucleotide and hexanucleotide repeats, which do not alter the reading frame, have been correlated with skeletal morphology in dogs (8), mating behavior in prairie voles (9), and circadian rhythms in a number of species (2). The mutability of repeats, combined with their incremental effects on protein levels and activity, suggests they could be effective agents for evolutionary plasticity, a hypothesis reinforced by the overrepresentation of trinucleotide repeats (TNRs) in coding regions and their enrichment in genes for transcription factors and other regulatory proteins (10, 11).

The valuable role of microsatellite repeats as “tuning knobs” of evolution comes with a cost (12). At more than 20 loci in the

human genome, expansions of TNRs trigger human disease (13). Expanded TNRs are very unstable in germline and somatic tissues, usually with a bias toward further expansion. Their mutagenesis is stimulated by most DNA transactions—replication, repair, recombination, and transcription—that expose single strands of repeats, allowing them to form non-B DNA secondary structures and slipped-strand duplexes that are thought to be common intermediates in pathways leading to repeat instability (14–17).

Recent investigations into the instability of expanded TNRs have revealed that the stress-responsive chaperone, heat shock protein 90 (HSP90), acts to maintain the stability of long TNRs in human cells by regulating double-strand break (DSB) repair (18, 19). When HSP90 is inhibited or knocked down, TNRs mutate more frequently. This effect of HSP90 on TNR mutability adds to a growing list of links between HSP90 and genomic events (20). Environmental stresses, which divert HSP90 from supporting its many client proteins to the emergency role of repairing damaged proteins, can trigger HSP90-linked genomic events (21) and could induce mutagenesis of TNRs.

Stress-induced mutagenesis (SIM) is a well-defined phenomenon in bacteria and yeast, where it has been shown that a variety of stresses relax the fidelity of DNA repair, enhancing mutagenesis and often increasing the odds of survival (22, 23). SIM also is strongly implicated in the increased mutation frequency associated with cancer cells growing in a hypoxic tumor microenvironment (24). These well-studied examples of SIM and the link between HSP90 and TNR instability raise the critical

Significance

Environmental stress induces complex cellular responses that improve the odds of survival. One effect of the cellular stress response is to relax the fidelity of DNA repair, with the counterintuitive strategy of increasing mutagenesis to improve fitness. Because microsatellite repeats, which are intrinsically highly mutable, serve as tuning knobs for evolutionary change, we asked whether environmental stress increased their mutagenesis. We found that cold, heat, hypoxic, and oxidative stresses induce trinucleotide repeat (TNR) mutagenesis, in a way that depends on stress response factors and apparently involves DNA rereplication. Because TNRs are overrepresented in gene-regulatory proteins, stress-induced TNR mutagenesis may provide a path for the environment to subtly alter gene regulatory networks—with attendant changes in cell behavior—during development, disease, and evolution.

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Fig. 2. Effects of siRNA knockdown of SRFs and DNA repair proteins on stress-induced GFP⁺ cells. (A) Knockdown of SRFs. Cells were plated 2 d before stress, and siRNAs were transfected 1 d before stress. GFP⁺ cells were analyzed after 3 d of recovery. Frequencies of GFP⁺ cells after stress were normalized to unstressed cells that were treated with an ineffective vimentin-siRNA. The frequency of GFP⁺ cells in unstressed control cells was 0.19%. (B) Knockdown of DNA repair proteins. Experiments in A and B were carried out at the same time; the vimentin controls are the same in both and are repeated for clarity. Results were measured in three independent experiments, each with three replicates. Error bars represent SDs. *** $P < 0.001$ versus control, based on Student's two-tailed t test.

pathways generate GFP⁺ cells, it does not define the mechanism by which CAG repeat tracts are altered. To gain insights into proximate cause of SIM of CAG repeats, we tested the involvement of several DNA metabolic processes, including transcription, mismatch repair (MMR), nucleotide excision repair (NER), base excision repair (BER), and replication.

We found that induction of transcription, which efficiently destabilizes CAG repeat tracts in human cells (30, 31, 33, 34), is not required for stress-induced production of GFP⁺ cells. Heat, cold, hypoxic, and oxidative stress induced the same four- to fivefold increase in GFP⁺ cells regardless of whether transcription of the GFP gene was induced by doxycycline before stress and occurred throughout the 3-d recovery period (Fig. S4A) or was induced only for the final day of recovery (Fig. S4B).

To test the potential roles of MMR, NER, BER, and replication, we knocked down key components in each process—MutS homolog 2 (MSH2) for MMR, xeroderma pigmentosum, complementation group A (XPA) for NER, 8-oxoguanine glycosylase (OGG1) for BER, and flap endonuclease 1 (FEN1) and petite integration frequency 1 (PIF1) for replication. Knockdown efficiencies ranged from 72–96% (Table S1). Knockdown of MSH2 and XPA had no effect on the stress-induced levels of GFP⁺ cells (Fig. 2B), suggesting that MMR and NER are not involved. We also showed that siRNA knockdowns of MutL homolog 1 (MLH1) (MMR) and Cockayne syndrome B (CSB) (NER) had no effect on heat- and hypoxic stress-induced production of GFP⁺ cells (Fig. S5). Similarly, knockdown of OGG1 did not influence stress-induced GFP⁺ cells, except for oxidative stress, which creates the lesions targeted by BER (Fig. 2B). In contrast, knockdown of the replication factors FEN1 (flap endonuclease) and PIF1 (DNA helicase) gave a consistent reduction in GFP⁺ cells in all four stresses (Fig. 2B), suggesting

that stress-induced CAG mutagenesis is associated with a replication process.

Stress-Induced Rereplication May Induce TNR Mutagenesis. More than 40 years ago, Schimke and colleagues (35) showed that hypoxia-induced amplification of the dihydrofolate reductase (*DHFR*) gene involved DNA rereplication, which is the inappropriate firing of replication origins more than once in a cell cycle. To investigate the involvement of rereplication in SIM of CAG repeats, we initially tested whether our stress protocols increased *DHFR* gene amplification, which we detected by the production of methotrexate-resistant colonies (35). As shown in Fig. S6, cold, hypoxic, and oxidative stresses induced a 10- to 15-fold increase in methotrexate-resistant colonies, consistent with gene amplification (35). Moreover, the percentage of cells with >4 C-value (C) DNA—an indicator of rereplication—increased from less than 5% in unstressed cells to more than 20% in cells exposed to cold, heat, hypoxic, and oxidative stress (Fig. 3A and Fig. S7). [The percentage of cells with >4C DNA did not increase with starvation stress (Fig. S3).] For hypoxia, we showed that knockdown of either HIF1 or HIF3 significantly reduced the stress-induced increase in the percentage of cells with >4C DNA (Fig. S8). As was the case with GFP⁺ cells, the cells with >4C DNA increased most prominently in the recovery period after stress (Fig. 3A), in agreement with Schimke's original observations (35).

If rereplication generates GFP⁺ cells, then treatments that reduce rereplication also should reduce the frequency of stress-induced GFP⁺ cells. Because overexpression of the origin-licensing factor chromatin licensing and DNA replication factor 1 (CDT1) increases the proportion of cells with >4C DNA (36, 37), we reasoned that knockdown of CDT1 would reduce the number of

Fig. 3. Stress-induced repeat instability and rereplication. (A) Proportion of cells with >4C DNA content with increasing time of recovery. (B) Effects of CDT1 knockdown on percentages of cells with >4C DNA content (Left) and percentage of GFP⁺ cells (Right) after various stresses. Vimentin siRNA is a control siRNA that has no effect on cells. (C) Effects of aphidicolin treatment on the percentages of cells with >4C DNA content (Left) and the percentages of GFP⁺ cells (Right). All results were averaged from three independent experiments, each with three replicates. Error bars represent SDs. Statistical significance was determined by Student's two-tailed t test: ** $P < 0.01$; *** $P < 0.001$.

cells with >4C DNA. As shown in Fig. 3B, siRNA-mediated knockdown of CDT1 (85% knockdown efficiency; Table S1) eliminated not only the stress-induced increases in the proportion of cells with >4C DNA content but also the stress-induced increases in GFP⁺ cells. To test whether rereplication, independent of environmental stress, can induce GFP⁺ cells, we treated unstressed cells with aphidicolin, which increases the proportion of cells with >4C DNA content (38). As shown in Fig. 3C, aphidicolin increased the percentages of GFP(CAG)₈₉ cells with >4C DNA content and also the percentages of GFP⁺ cells. These effects are not caused by the direct inhibition of DNA synthesis, because GFP⁺ cells (and cells with >4C DNA) increased most dramatically during the recovery period after aphidicolin was removed (Fig. S9). Moreover, both effects were eliminated by knockdown of CDT1. We conclude that stress-induced DNA rereplication is likely to be responsible for generating GFP⁺ cells.

Discussion

In this study, we set out to determine whether environmental stress induces mutagenesis of TNRs in human cells. Using a long CAG repeat tract in a GFP minigene, we showed that cold, heat, hypoxic, and oxidative stresses increased mutagenesis of the CAG repeat about fourfold relative to unstressed cells. For each of these stresses, we were able to eliminate the induced mutagenesis by knocking down individual SRFs that mediate the stress response in human cells (28, 29). By using a selectively neutral reporter to show that environmental stresses can increase the mutagenesis of CAG repeats in a way that is blocked by interfering with the stress response, we have met the principal criteria for establishing SIM (39). We conclude that SIM of TNRs occurs in human cells.

To identify the pathway by which SIM causes mutations at CAG repeat tracts, we initially tested several established causes of TNR instability. We eliminated transcription as a possible mechanism by showing that mutagenesis was induced to the same extent regardless of the transcriptional status of the reporter gene. Using siRNA knockdowns of key pathway components, we showed that MMR and NER, whose normal function actively promotes TNR instability (40), were not required for stress-induced TNR mutagenesis. This outcome is not surprising, because the stress response typically depresses the activity of these DNA-repair pathways (29), an effect that should reduce TNR mutations instead of increasing them if these pathways were critical for mutagenesis. We found that the role of BER was confined to mutagenesis induced by oxidative stress, which introduces the types of DNA damage commonly repaired by BER. Only with the knockdown of the replication factors FEN1 and PIF1 did we achieve a consistent reduction in SIM of TNRs, suggesting that TNRs were mutated via a replication-related mechanism.

In a series of classic studies, transient hypoxia was shown to trigger amplification of the *DHFR* gene via a mechanism linked to rereplication during the recovery phase (35, 41). In accord with those studies, we showed that cold, hypoxic, and oxidative stresses induced gene amplification in our cells. We also showed that the increase in stress-induced TNR mutagenesis during the recovery phase was accompanied by an increase in cells with >4C DNA content, a hallmark of rereplication. Knockdown of SRFs blocked both stress-induced TNR mutagenesis and DNA rereplication. Moreover, we were able to eliminate stress-induced TNR mutagenesis by knocking down the origin-licensing factor CDT1, and this knockdown also blocked rereplication. Finally, we showed that direct induction of DNA rereplication by aphidicolin promoted TNR mutagenesis in the absence of environmental stress. Knockdown of CDT1 also blocked both aphidicolin-induced TNR mutagenesis and rereplication. We conclude that stress-induced TNR mutagenesis likely involves

rereplication, a process that previously has not been linked to TNR instability.

We do not know how rereplication might induce TNR mutagenesis. However, the mutations to the CAG repeat tracts in the GFP⁺ cells—46% contractions and 54% indels—offer a clue. In most of our previous characterizations of CAG repeat instability, using the GFP-based assay or our HPRT selection system, we observed primarily simple contractions of the repeat tract; only about 5% were indels (30, 33). Only when we introduced DSBs into the CAG repeat tract, using zinc-finger nucleases (42), did we observe a high frequency of indels (44%). Thus, DSBs, which are introduced into the genome during recovery from hypoxia (43), may contribute to stress-induced TNR mutagenesis, perhaps during resolution of the aberrant chromosomal structures generated by rereplication.

In bacteria, where it was first defined, and in yeast and human cancer cells, SIM has been shown to cause several kinds of genomic changes, including indels and changes in the number of repeats in a run (23). A major pathway for SIM in bacteria and cancer cells occurs as a result of error-prone repair of DSBs (29, 44). In bacteria, mutagenic repair of DSBs involves an error-prone DNA polymerase called into play by the stress response; in mammalian cells, error-free homology-directed repair of DSBs is down-regulated, enhancing the contribution of error-prone nonhomologous end joining to the mutagenic process. Whether error-prone polymerases or error-prone end joining, if either, is used in stress-induced TNR mutagenesis remains to be determined.

The pathway for stress-induced TNR mutagenesis shows intriguing parallels with pathways that lead to cancer. Cancer cells often further their survival and growth in hostile environments by increasing the expression of key SRFs such as CIRP, HSF1, HIF1, and NRF2 (45–48). One of the consequences of SRF expression in hypoxic cancer cells is increased mutagenesis (29), similar to the SIM of TNRs observed here. The proximate cause of mutagenesis, rereplication, which we observe here during recovery from stress, is thought to play a central role in the genomic instability leading to cancer progression (49, 50). Studies from a variety of sources show that rereplication increases aneuploidy, chromosome fusions, and DNA breaks, which are common tumor-cell markers (51). Pathways for tumorigenesis and TNR mutagenesis may intersect at TNR repeats, which are more prevalent in cancer-related human genes, and may make those genes more prone to mutagenesis (52).

Collectively, our study outlines a pathway for stress-induced TNR mutagenesis in human cells. This SIM pathway depends on stress-response factors that are known to mediate responses to environmental stress and induce DNA rereplication, which is likely the proximate cause of TNR mutagenesis. This pathway may impact abnormal cells as they evolve metastatic potential; however, it also may affect normal cells, even differentiated cells, as they encounter stresses in their environment or during development (53, 54). SIM of TNRs (and other microsatellites) constitutes an effective way to promote incremental adjustments in protein activity and gene expression, with attendant changes in cell behavior.

Materials and Methods

Cell Culture. GFP(CAG)₈₉ cells, derived from T-REX HEK293 cells, were grown at 37 °C with 5% CO₂ in DMEM (Gibco) supplemented with glucose, 10% (vol/vol) FBS (HyClone; Thermo Scientific), and 1% minimal essential medium nonessential amino acids (Gibco). Cells were trypsinized using 0.25% trypsin-EDTA (Gibco) for cytometry and passaging.

GFP Assay. Construction and characterization of the GFP(CAG)₈₉ and GFP(CAG)₀ HEK293 cell lines are described elsewhere (30). Briefly, the GFP(CAG)₈₉ cell line carries a GFP minigene, which is under the control of the inducible CMV/TetO₂ hybrid promoter (the cytomegalovirus immediate early promoter plus two tetracycline operator 2 sites). To measure GFP fluorescence, GFP

expression was induced with doxycycline (4 μ g/mL) (Clontech), cells were trypsinized, pelleted, and resuspended in 1% paraformaldehyde in PBS (Invitrogen), and then were analyzed by flow cytometry (BD LSR II flow cytometer; BD Biosciences). Induced levels of GFP expression are about 90-fold above background, and GFP expression reaches a plateau after about 20 h (30). GFP⁺ cells were defined by the gate shown in Fig. S1, which cleanly separates the main peaks of fluorescence in GFP(CAG)₈₉ cells and GFP(CAG)₀ cells. All cells isolated from the right side of this gate have changes in the CAG repeat tract. In our previous study using similar gates, contractions left 7–35 CAG repeat units in the repeat tracts remaining in GFP⁺ cells (30). In all cases, frequencies were calculated as the number of GFP⁺ cells divided by the total number of cells in the analyzed population.

Environmental Stress. GFP(CAG)₈₉ cells were plated in six-well plates (500,000 cells per well) and were incubated at 37 °C for 1 d. Doxycycline (4 μ g/mL) then was added to induce transcription fully. Stress was administered to one set of GFP(CAG)₈₉ cells, and a second set was maintained at 37 °C to serve as the unstressed control. For cold stress, cells were incubated at 30 °C for 24 h (55). For heat stress, cells were incubated at 44 °C for 24 h. For hypoxia, cells were incubated for 48 h in 1% O₂ in a hypoxic chamber (56). For oxidative stress, cells were treated with 0.5 mM H₂O₂ in 5% (vol/vol) FBS medium for 15 min (57). At the end of the stress period, fresh medium was added, and

the cells were incubated at 37 °C until harvested for analysis. GFP⁺ cells were analyzed by flow cytometry at 0, 2, 3, and 7 d after stress.

DNA Content. Cells were analyzed for DNA content at 0, 18, and 72 h after stress. Cells were trypsinized and resuspended in 1 mL of 1% paraformaldehyde in PBS overnight at 4 °C. After pelleting, 1–2 $\times$ 10⁵ cells were resuspended in 1 mL DAPI mix (PBS with 0.1% Triton-X and 0.1% DAPI stock) and incubated at room temperature for 2 h in the dark. Cells were analyzed on a BD LSRFortessa cell analyzer (BD Biosciences), using stringent gates to ensure that only singlet cells were counted. To identify cells with >4C DNA content, we used a gate just to the right of the G2M (4C DNA) population in unstressed cells (Fig. S7). Cells beyond this gate were defined as having >4C DNA. The percentage of cells with >4C DNA was calculated by dividing the number of cells with >4C DNA by the total number of singlet cells in the population.

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