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Persistent Genetic Instability in Cancer Cells Induced by Non-DNA-damaging Stress Exposures[1]

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Abstract

A hallmark of cancer cells is their pronounced genetic instability, which has been implicated in both tumor development and negative treatment outcomes. Recently, it has been reported that ionizing radiation may induce a persistent state of hypermutability in mammalian cells that lasts for many (>30) cell divisions. In this study, we examined whether other stress signals (both DNA-damaging non-DNA-damaging) can initiate a similar process. We show that persistent genetic instability was induced by nongenotoxic stress exposures such as heat treatment, serum starvation, or the tumor microenvironment, as well as genotoxic stresses such as ionizing radiation and exposure to hydrogen peroxide. Progeny of 10–20% of surviving cells exhibited persistent and pronounced genetic instability at both an artificially transfected gene and a genomic minisatellite locus 23 cell divisions after the initial exposure. Stress-induced persistent genetic instability may be a general response of tumor cells to a wide range of genotoxic or nongenotoxic stress conditions.

Introduction

Genetic instability has been implicated prominently in tumor development. The main evidence comes initially from the discovery of chromosomal aberrations and later from the identification of numerous mutations in key genes, oncogenes, or tumor suppressor genes, among almost all types of cancer. A tremendous amount of progress in the past 30 years has shed much light on how these mutations can affect a normal cell biochemically and/or genetically and render it carcinogenic. However, no definitive explanation is available as to how the mutations occur. It has been generally attributed to cellular exposure to chemical or physical agents that can damage DNA or random errors committed by the cellular DNA replication machinery (1). According to this theory, the errors induced by the DNA-damaging agents or replication errors cause the cells to accumulate the "right" set of mutations in key genes over time to become carcinogenic eventually.

Although there is clear evidence that supports the prominent roles DNA-damaging agents play in carcinogenesis, a careful examination by authors of several earlier reports (2–4) led to a major dilemma: the mutation rate of cancer cells (10^{-7}–10^{-5} cells/division; Ref. 1) cannot account for the multiple (seven to eight in the case of colon cancer) mutations that are required to occur in a single cell for the cell to become tumorigenic (5). A "mutator" phenotype, therefore, has been proposed to be necessary for cancer development. The recent discovery that both sporadic and genetically predisposed colon cancer cells harbor as many as 10^5 mutations individually further reinforces this idea. However, there is no direct evidence for the existence of a hypermutable state in cancer cells. The only exception occurs in cells that are deficient in mismatch repair (6), although most cancer cells exhibit normal repair capacity when examined.

Recently, it has been observed that ionizing radiation can induce a persistent state of hypermutability in mammalian cells that lasts for many cell divisions after initial exposure (7, 8). Because of the potential importance of this discovery, we examined whether other stress signals, especially those that are non-DNA-damaging, can initiate a similar process in a murine tumor cell line. Our results indicate that stress-induced persistent genetic instability may be a general response of cancer cells surviving environmental stress exposure, whether genotoxic or nongenotoxic.

Materials and Methods

Cell Line and Tissue Culture Condition. The 4T1 cell line used in this study was kindly provided by Dr. Fred Miller of the Michigan Cancer Foundation. It is derived from a spontaneously arising mammary carcinoma in a Balb/c mouse (9). The cells were grown in DMEM with 10% heat-inactivated fetal bovine serum and penicillin (50 units/ml), and streptomycin (50 μg/ml).

EGFP[4] Transduction and Selection for Stable Clones. The 4T1 cells were transfected with a plasmid, pEGFP-N1, that constitutively expresses an enhanced version of the green fluorescence protein (Clontech, Palo Alto, CA; Ref. 10). The liposome DMRIE from Life Technologies, Inc. (Gaithersburg, MD) was used. A protocol recommended by the manufacturer was followed. G418 selection at 400 μmg/ml started 2 days after transfection. Two to 3 weeks later, colonies that grew out in Petri dish cultures were examined for GFP expression under a fluorescence microscope. Those that appeared to have robust GFP expression were picked, trypsinized, and expanded for Southern blot analysis. A single subclone with a single copy of GFP inserted at one site in the 4T1 genome was selected for subsequent experiments.

Stress Exposure of the Cells. For ionizing radiation exposure, ~10,000 tumor cells were plated in each 10-cm Petri dish 1 day before treatment. The dishes were then irradiated with 12 Gy of γ-rays at a dose rate of 8 Gy/min by use of a Cs-137 irradiator (J. L. Shepard and Associates). Surviving colonies were then selected for subsequent expansion and analysis. For heat exposure, ~10,000 cells were plated into each 10-cm Petri dish 1 day before the treatment. The plates were completely wrapped with Parafilm before immersion. Only one-third of the whole dish was submerged, because there was only 5 ml of medium in the P-100 dish. The dishes remained submerged in the water bath with temperature controlled at 45°C for 30 min. Afterward, the dishes were thoroughly wiped in 70% ethanol and removed from Parafilm and then placed back into the incubator. The surviving colonies were then selected for subsequent expansion and analysis. For H_2O_2 exposure, 10,000 cells were plated into each Petri dish to be treated 24 h before treatment. H_2O_2 was then added to the dish at a concentration of 500 μM. The cells were exposed for 4 h, and H_2O_2 was removed together with the medium. The surviving colonies were then selected for expansion and analysis. For serum starvation treatment, cells grown to 80% confluence were incubated in serum-free medium for 7–8 days until >95% of the cells were killed. Normal fetal bovine serum-comple-

[4] The abbreviation used is: EGFP, enhanced green fluorescent protein.

mented medium was then added, and surviving colonies were selected for expansion and analysis. For growth *in vivo*, 10^6 4T1-GFP cells were injected s.c. into the right hind leg of syngeneic mice. Two to 3 weeks later, tumors with diameters of 1.0–1.5 cm formed and were excised. Tumors were then minced and digested with collagenase and plated out at low density. GFP-expressing clones were then selected for expansion and analysis.

Fluorescence Microscopy. Visualization of GFP expression was carried out on a Zeiss Axioscope equipped with a 3CCD color camera. A computer equipped with a frame grabber was connected to the camera to capture images on-line. To visualize GFP-expressing cells, epifluorescence (xenon arc source and FITC filter) with or without concomitant *trans*-illumination (with a 40-W tungsten source) was used.

Isolation of Genomic DNA and Southern Blot Analysis. Isolation of genomic DNA and Southern blot analysis was carried out according to an established protocol (11). To carry out analysis for the inserted *GFP* gene, 10 μg of each sample were digested with *Bam*HI (New England Biolabs, Inc. Beverly, MA) and electrophoresed at 3.3 V/cm for 6 h in 1% agarose gel. To conduct minisatellite analysis, 10 μg of each sample of DNA were digested with *Hin*fI and separated in 0.8% agarose gels at 3.3 V/cm for 32 h. Then the gels for both GFP and minisatellite analysis were soaked in 0.25 M HCl for 20 min. Afterward, the DNA was transferred onto Nytran nylon membranes (Schleicher & Schull). The membranes were then UV irradiated and prehybridized for 3 h at 65°C in 6× SSC, 5× Denhardt's solution, and 1% SDS. They were then hybridized with ^{32}P-labeled probes. For EGFP, the GFP encoding fragment from the pEGFP-N1 was used as the probe. For minisatellite analysis, a minisatellite probe, M, derived from the mouse MHC sequences (12), was used. After hybridization, the membranes were washed three times in 6× SSC for 15 min at room temperature and then autoradiographed.

Results

Visualization of Persistent Genetic Instability at an Artificially Inserted *GFP* Gene Locus. To monitor genetic instability in a direct and accurate manner, a murine mammary adenocarcinoma cell line, 4T1, was transduced with the EGFP-N1 plasmid, which encodes the *EGFP* gene under the control of the cytomegalovirus immediate-early promoter. After selection for stable expression of the *GFP* gene, a subclone was obtained that expresses GFP constitutively. All of the cells appeared uniformly green under a fluorescence microscope (Fig. 1*a*). Southern blot analysis indicated that a single copy of the plasmid had integrated into the host genome (Fig. 1*b*). Therefore, a loss of green fluorescence in any cells from this subclone would indicate a loss of gene expression, which in turn would indicate a potential mutation at the integrated *GFP* gene. The exact nature of the mutation can easily be determined by Southern blot analysis. Cells were subjected to exposure to various genotoxic and nongenotoxic stress conditions. There were six experimental groups: (*a*) a control group
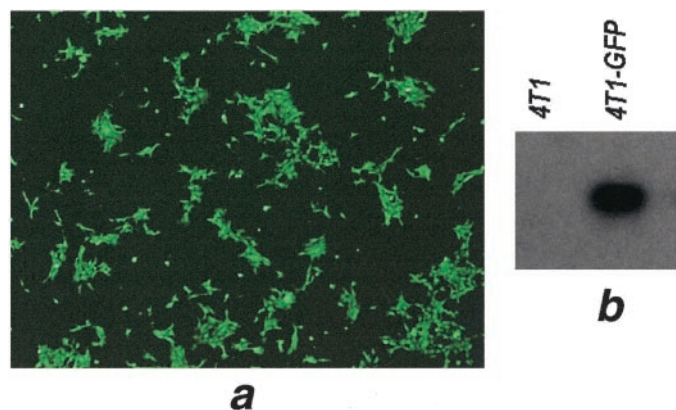

Fig. 1. Stable transduction of the *GFP* gene into the 4T1 cell line. *a*, fluorescence photomicrograph of stably transduced 4T1 cells. *b*, Southern blot analysis of the transduced *GFP* gene. A single band was observed to be present on the Southern blot. Laser densitometry analysis indicate a single copy was inserted into the genome of the 4T1 cell.
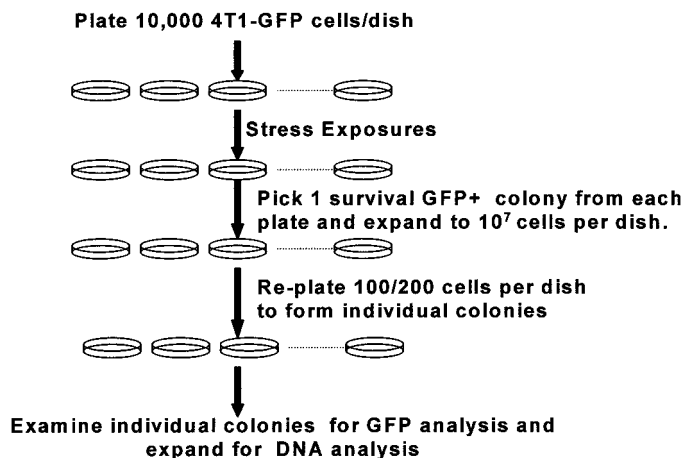

Fig. 2. A selection scheme for clones of 4T1-GFP cells that have survived different exposure treatments.

with no exposure; (*b*) two groups subjected to genotoxic exposures: ionizing radiation, 12 Gy, which kills >95% of the cells; and hydrogen peroxide exposure, 0.5 mM for 24 h, which kills >95% of the cells; and (*c*) three groups subjected to nongenotoxic exposures: hyperthermia, 45°C for 30 min, which kills over 95% of the cells; serum starvation, which deprives serum for 8–10 days and kills >95% of the cells; and growth as a tumor *in vivo* for 14–21 days and subsequent growth in cell culture.

Surviving clones from the exposed cell cultures were then selected according to a scheme shown in Fig. 2. After exposure to various stress conditions, 12–20 of the surviving colonies from each exposure group were selected. These colonies were selected based on the fact that they appeared to be homogeneously green under a fluorescence microscope and therefore should possess an intact *GFP* gene at the time of selection. The colonies were then expanded to ~10^7 cells each, or ~23 cell divisions after the initial stress exposure. Cells from each clone were then separately plated out at 100–200 cells/dish for growth into individual colonies. After 14 days, each colony in a Petri dish was carefully scrutinized under a fluorescence microscope for its GFP expression. There were three categories of expression patterns: the first were those that were homogeneously green (Fig. 3, *a* and *b*), indicating the integrity of the *GFP* gene; those that were completely dark (not shown), indicating a potential loss of the *GFP* gene; and those that had a chimeric pattern of GFP fluorescence (Fig. 3, *c–f*), indicating a mixture of cells with or without GFP expression. The mixed colonies are especially important because they indicate an on-going process of genetic instability at the *GFP* locus.

Table 1 is a summary of the results. It is clear that in the control group, all of the colonies are homogeneously green, indicating a lack of mutations at the inserted *GFP* gene locus. In contrast, all stress-exposed groups possess colonies that were either mixed or completely dark, indicating mutations in the *GFP* gene. Because all of the colonies in Table 1 were derived from individual cells that had been exposed to the stress conditions at least 23 cell divisions earlier, the presence of mixed colonies unequivocally indicates the continued presence of genetic instability among these clones. The pattern of mixed colonies was diversified, with some demonstrating an almost even split between dark and fluorescent cells (Fig. 3, *c* and *d*) and most demonstrating a more mosaic phenotype (Fig. 3, *e* and *f*).

To determine the exact cause for the loss of GFP expression in the dark cells, colonies with the mixed GFP phenotype were further subcultured (plated with 100/200 cells per 10-cm Petri dish), and the colonies with no GFP expression were isolated and expanded. In all cases where the dark colonies were subcultured, the cells were able to

Fig. 3. Fluorescent photomicrographs of 4T1-GFP clones with various GFP expression patterns. *a* and *b*, clones with no loss of GFP expression. *c–f*, "chimeric" clones with partial loss of GFP expression.
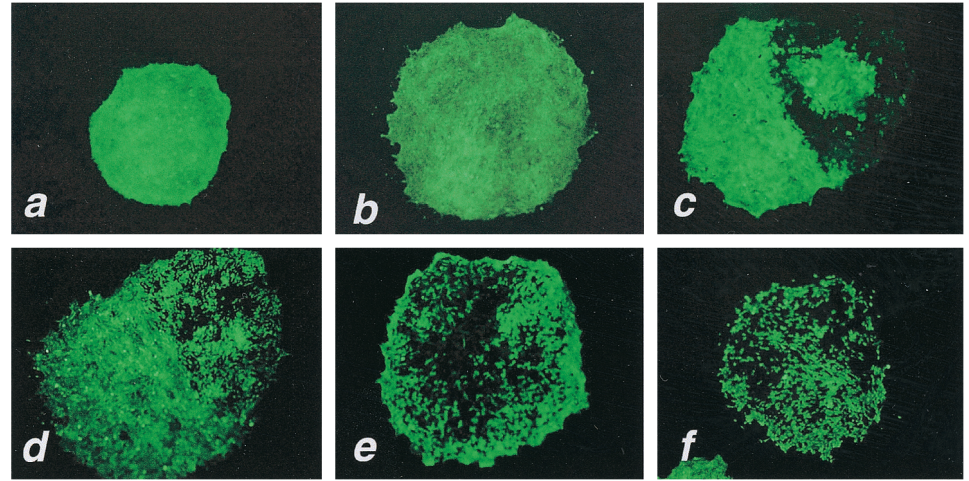
Table 1 *The fraction of progeny colonies exhibiting various green fluorescence patterns 23 generations after exposure to various nongenotoxic and genotoxic stress exposures*

	Control	Nongenotoxic exposure			Genotoxic exposure	
		Heat	Serum starvation	*In vivo* growth	H_2O_2	Radiation
Bright	812	486	410	295	535	536
Dark	0	11	27	24	12	86
Chimeric	0	36 (6.8%)[a]	68 (13.5%)	24 (7.0%)	39 (6.7%)	75 (10.8%)
Total	812	533	505	343	586	697

[a] Numbers in parentheses, fraction of chimeric colonies among all colonies examined.

replicate, although some colonies displayed a significantly slower proliferation rate than parent cells (doubling times increased from 28 h for parent cells to 50 h for some treated clones). PCR was used to amplify the *GFP* gene from the dark clones with no success, indicating a loss of the *GFP* gene genomically. Southern blot analyses were subsequently carried out for genomic DNA derived from these dark cells by use of the GFP coding sequence as the probe. Table 2 is a summary of the results. It is apparent that all of the dark colonies had a deletion involving the inserted *GFP* gene.

The most significant finding from these data is that the persistent genetic instability at the inserted *GFP* locus was observed in all of the stress exposure groups, irrespective of whether the stress is directly DNA-damaging (radiation, H_2O_2) or non-DNA-damaging (heat treatment, serum starvation, and *in vivo* tumor growth) in the conventional sense. This is striking because pronounced, persistent genetic instability such as that represented here has never been described before as a consequence of nongenotoxic stress exposures, although there have been a few reports demonstrating transiently elevated mutation frequency in the tumor microenvironment or under serum-deprived conditions (13, 14).

Another important question is whether there is any dosage threshold for the observed genetic instability. Although we did not carry out experiments to systemically determine the minimal dosage that was required to induce persistent genetic instability, we did treat the cells with lower doses of ionizing radiation (4 and 6 Gy, which kills about 60 and 80% of the cells, respectively) and heat (44°C for 30 min, which kills only 50% of the cells). Under these less lethal conditions,

genetic instability at the *GFP* locus was still observed. There did not appear to exist a linear relationship between dose and the percentage of genetically unstable clones. Both 4 and 6 Gy of ionizing radiation produced a similar percentage (10–20%) of genetically unstable cells among the progeny cells. Both the lack of linearity and the percentage of genetically unstable clones are similar to what has been reported in an earlier study (15). Heating at 44°C for 30 min produced a much lower (0.8%) frequency of mutation.

Persistent Genetic Instability at an Endogenous Minisatellite Locus. To determine whether the observed genetic instability is present only at the artificially inserted *GFP* gene, a minisatellite probe, M, derived from the mouse MHC sequences (12), was used to probe a subset of the colonies that showed a loss of the *GFP* gene from the stress-exposed cells. Such minisatellite probes, which detect multiple repetitive sequences at the same time, are a powerful tool for the determination of gross DNA changes at multiple loci (11, 16, 17). Again, significant DNA rearrangements at the minisatellite loci detected by the M probe were observed among both the genotoxic and nongenotoxic stress-exposed groups (Table 3 and Fig. 4). The most common changes were deletions of certain fragments (Fig. 4, *Lanes 2, 3, 8,* and *11*). There are also new bands (Fig. 4, *Lane 6*). Table 3 is summary of detected minisatellite rearrangements among different subclones from different groups. The prevalence of the minisatellite mutations is significant, with 16–30% of the randomly picked colonies possessing them. This rate of mutation is substantially higher than that observed for genes in conventional mutagenesis studies, although it is similar to what has been observed for radiation-induced, persistently hypermutable mammalian cells (15).

Discussion

The existence of an inducible state of genetic instability in mammalian cells is highly significant. Previously, such a phenomenon has been demonstrated clearly at the chromosomal and DNA level for ionizing radiation (7, 18–21). Our data here indicate that it may be a general phenomenon for cancer cells under environmental stress. Of

Table 2 *Status of the GFP gene in 4T1 colonies with loss of GFP fluorescence*

Treatment group	Heat	Serum starvation	Growth in tumor	Radiation	H_2O_2
Deletion[a]/Total clones[b]	16/16	19/19	12/12	10/10	16/16

[a] Number of clones with one or more changes in their banding patterns on Southern blots.

[b] Total number of clones examined by Southern blots in each stress exposure.

Table 3 *The fraction of progeny colonies exhibiting mutant minisatellite patterns on the probe M-derived Southern blots 23 generations after exposure to various stress conditions*

		Nongenotoxic			Genotoxic	
Treatment group	Control	Heat	Serum starvation	Growth in tumor	Radiation	H_2O_2
Mutant[a]/Total clones[b]	0/24 (0%)	3[c]/16 (19%)	4[d]/19 (16%)	2[e]/12 (17%)	3[f]/10 (30%)	3[g]/16 (19%)

[a] Number of clones with change(s) in their banding patterns on Southern blots.
[b] Total number of clones examined by Southern blots in each stress exposure group.
[c] Three losses of existing bands.
[d] Three lost existing bands and one with new band.
[e] Two lost existing bands.
[f] Three losses of existing bands.
[g] One new band and two losses of existing bands.

particular importance is that both genotoxic and nongenotoxic stress can activate this process. Such pronounced genetic instability may have profound implications in many important biological processes. It suggests the existence of an inducible, highly dynamic state of the cancer cell genome that may underlie many aspects of cancer initiation and development. It is now generally recognized that multiple (*e.g.,* seven to eight for colon cancers) mutations at key oncogenes/ tumor suppressor genes are necessary for a normal cell to become tumorigenic (5, 22). In reality, it has been estimated that at least 11,000 individual DNA mutations exist in a single carcinoma cell in sporadic or genetically predisposed colorectal tumors (6, 23). Chromosomal mutations occurring at the frequency of 10^{-2}/chromosome/ cell division have also been observed in colorectal tumors. It has been proposed that this genetic instability is likely to be a cause rather than the result of the carcinogenic process (24, 25).

The mechanisms involved in causing such persistently increased frequencies of mutations at the DNA level are unclear, although a checkpoint gene *hBUB1* has been implicated for chromosomal instabilities (25). The sporadic, random accumulation of mutations through defects or errors in repair mechanisms (generally yielding mutation rates ranging from 10^{-7} to 10^{-5}/cell/division; Ref. 1) could not account for the occurrence of seven to eight oncogene/tumor suppressor mutations in the same cell, such as has been observed in many tumor types (2, 5, 22, 26). Furthermore, it is unlikely that the high frequency of genetically unstable clines (10–20%; Tables 1 and 3) we have observed could be explained by the induction of mutations in a specific mutator gene or set of genes in the cells surviving exposure to stress. The persistently increased mutation rate resulting from a

stress-inducible state of persistent genetic instability similar to that reported in this study could, however, account for the accumulation of multiple mutations in cancer cells. Perhaps triggered by exposure to environmental mutagens or abnormal physiological conditions, this hypermutable state could cause mutations at many genomic loci in a normal cell. Because it can persist for many generations, critical mutations will emerge in a Darwinian fashion (25) over time to render the cell tumorigenic. That such a phenomenon may be involved in the early stages of carcinogenesis is evidenced by the occurrence of multiple mutations in colonic polyps early in tumor progression (23). It is also very possible that this highly mutagenic process is active during the later stages of tumor development, perhaps even being perpetuated and sustained by the tumor microenvironment, which can be much harsher than the normal physiological conditions. An earlier report (13) and results from this study demonstrate this possibility. Finally, it is possible that the high rate of genomic deletion we observed for the *GFP* gene and the endogenous minisatellite locus are attributable to the fact that they are located in regions of the genome that is especially susceptible to DNA insertion or deletions (this may be the reason why GFP was inserted there in the first place). Our present experiments cannot determine whether this is true. However, examination of transgenic cells where the *GFP* gene is inserted at known loci with normal mutation rates (*e.g.*, the *HPRT* gene; Ref. 27) may help answer these questions.

In summary, we have documented the existence of a general, stress-inducible genetic instability in cancer cells. Although the precise molecular mechanism is not clear, it is likely that the process is involved in the development of cancer.

Acknowledgments

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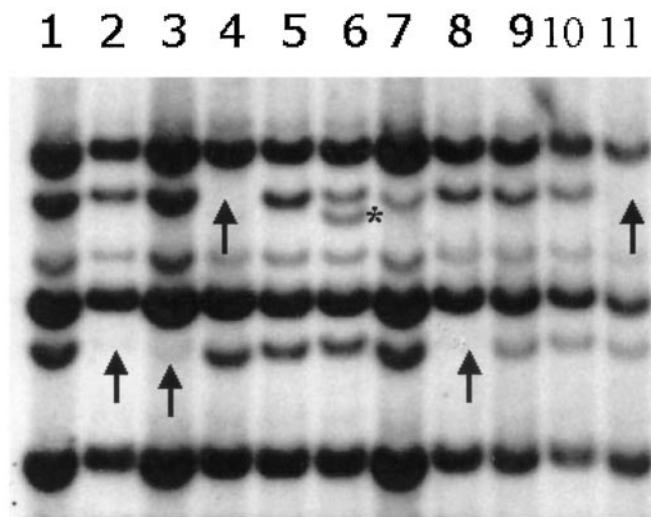
Fig. 4. Minisatellite analysis of stress-exposed clones. *Lane 1*, a control clone. *Lanes 2* and *3*, two radiation-treated clones. *Lanes 4* and *5*, two heat-treated clones. *Lanes 6* and *7*, two H_2O_2-exposed clones. *Lanes 8* and *9*, two serum-starved clones. *Lanes 10* and *11*, two clones derived from *in vivo* grown tumors. *Arrows*, loss of a band. *, appearance of a new allele.

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