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DNA Cleavage and Trp53 Differentially Affect SINE Transcription

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Abstract

Among the cellular responses observed following treatment with DNA-damaging agents is the activation of Short Interspersed Elements (SINEs; retrotransposable genetic elements that comprise over 10% of the human genome). By placing a human SINE (the Alu element) into murine cells, we have previously shown that DNA-damaging agents such as etoposide can induce both upregulation of SINE transcript levels and SINE retrotransposition. A similarly cytotoxic (but not genotoxic) exposure to vincristine was not associated with SINE activation. Here we demonstrate that multiple other genotoxic exposures are associated with upregulation of SINE transcript levels. By comparing the effects of similarly cytotoxic doses of the topoisomerase II inhibitors etoposide and merbarone, we confirm that DNA strand breakage is specifically associated with SINE induction. By evaluating transcription rate and RNA stability, we demonstrate that SINE induction by genotoxic exposure is associated with transcriptional induction and not with transcript stabilization. Finally we demonstrate that SINE induction by genotoxic stress is mediated by a Trp53-independent pathway, and in fact that Trp53 plays an inhibitory role in attenuating the transcriptional induction of SINE elements following exposure to a genotoxic agent. Together these data support a model in which initial DNA damage can trigger genomic instability due to SINE activation, a response which may be amplified in cancer cells lacking functional TP53.

INTRODUCTION

It is estimated that over one-third of the human genome is composed of DNA derived from retro-transposable genetic elements (Lander et al., 2001). Short Interspersed Elements (SINEs) and Long Interspersed Elements (LINEs) are the largest two classes of these elements. SINE and LINE elements replicate through RNA intermediates, increasing in copy number with each replicative cycle (Luan et al., 1993; Cost et al., 2002). SINE elements are transcribed by polymerase III, and reverse transcription and integration of the resulting noncoding RNA transcript appears to depend on the action of proteins encoded by LINEs (Dewannieux et al., 2003; Hagan et al., 2003). It is thought that SINE elements are entirely dependent upon the LINE machinery and other cellular factors for their retrotransposition. SINE elements are therefore considered nonautonomous retrotransposons in contrast to LINE elements, which encode many of the factors implicated in their replication. Recognition and retrotransposition of SINEs by LINE-encoded factors appears to be at least in part dependent on the strong sequence similarity of the 3' ends of SINEs and classes of LINEs that can promote their movement (Okada and Hamada, 1997). Subcellular localization may also facilitate SINE element retrotransposition. The Alu element, the most prevalent human SINE, is related to the 7SL RNA component of the signal recognition

particle (Ullu and Tschudi, 1984; Lander et al., 2001). Association between Alu RNA and components of the ribosomal translational machinery may place SINE elements in close proximity with nascent proteins, including newly translated LINE proteins (Sarrows et al., 1997). Multiple mechanisms may contribute to the ability of SINE elements to seemingly coopt LINE retrotransposition machinery.

While Alu elements are the predominant SINE element in the human genome, murine B1 and B2 elements comprise the largest percentage of murine SINE elements, each representing ~3% of the genome (Waterston et al., 2002). The B1 element, like the human Alu element, is derived from the 7SL RNA (Quentin, 1994a,b). B2 elements are derived from tRNA, as are most murine and human SINE elements with the exception of B1 and Alu (Schmid, 1998). Sequencing of various mammalian genomes has allowed for greater understanding of SINE and LINE evolution. While retrotransposons comprise similar percentages of the mouse and human genome (27 and 34%, respectively), analyses of the sequence variability among mobile elements suggest that these elements are amplifying at a higher rate in the murine genome than in the human genome.

The mechanisms regulating activation of SINE transcription have not been fully elucidated. Cellular stresses such as viral infection (Jang and Latchman, 1989; Panning and Smiley, 1994; Wick et al., 2003; Williams et al., 2004), heat shock, and inhibition of translation (Liu et al., 1995; Li et al., 2000) have been shown to increase human Alu transcript levels. Studies investigating transcription of murine B1 and B2 SINE elements have shown that they are similarly activated by viral infection, heat shock, and cyclohexamide (Fornace and Mitchell, 1986; Carey and Singh, 1988; Fornace et al., 1989; Price and Calderwood, 1992; Liu et al., 1995; Li et al., 1999; Wick et al., 2003; Allen et al., 2004; Williams et al., 2004). Recent work in our laboratory has shown that commonly used genotoxic chemotherapeutic agents such as the topoisomerase II inhibitor etoposide increase both human and murine SINE transcript levels and induce SINE retro-transposition (Rudin and Thompson, 2001; Hagan et al., 2003). We found that exposures to a variety of DNA-damaging agents, differing in mechanisms of action, were consistently associated with increases in intracellular SINE transcript levels.

Many DNA damage response pathways converge on common downstream effectors, most notably, on the transcriptional activator TP53. Following DNA damage, TP53 protein is stabilized. Both transcription-dependent and transcription-independent functions of TP53 contribute to cellular responses to genotoxic stress, including induction of cell cycle arrest, DNA repair pathways, and the intrinsic apoptotic pathway. Because of its central role in the cellular response to DNA damage, we hypothesized that TP53 might regulate the SINE transcriptional response observed following treatment with DNA-damaging agents. The purpose of this study was to determine which classes of cytotoxic agents could upregulate SINE transcript level, whether this upregulation was due to transcriptional induction or increased message stability, and to what extent this effect could be modulated by the murine homology of TP53, Trp53.

METHODS

Cell Lines

FL5.12 and derivatives generated from that cell line, FL5.x_L and FL5.x_L.DD, have been previously described (McKearn et al., 1985; Boise et al., 1993; Minn et al., 1996). P33-3 (Trp53 +/–) and P33-6 (Trp53 –/–) primary murine embryonic fibroblasts were a generous gift from Karen Frank at the University of Chicago, and were created as previously described (Donehower et al., 1992; Frank et al., 2000).

Cell Culture Conditions and Cytotoxic Exposure

FL5.x_L and FL5.x_L.DD cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 20 mM HEPES, 2 mM glutamine, 1 mg/ml geneticin, 50 μ M β -mercaptoethanol, 100 IU/ml penicillin, 100 μ g/ml streptomycin, and 10% WEHI-3B supernatant as a source of IL-3. Primary mouse embryonic fibroblast cells (P33-3 and P33-6) were cultured in DMEM high glucose supplemented with 10% fetal bovine serum, 20 mM HEPES, 2 mM glutamine, 100 μ M β -mercaptoethanol, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 IU/ml penicillin, and 100 μ g/ml streptomycin. All cells were cultured in 5% CO₂ at 37°C.

Chemotherapeutic concentrations (10 μ g/ml etoposide, 0.1 μ g/ml vincristine, 5 μ g/ml paclitaxel, 1 μ M topotecan, and 100 μ M merbarone) were chosen based on previous cytotoxicity data for the parental lines, and treatment at these doses yield similar levels of toxicity (Minn et al., 1995). Unless otherwise noted, cells were treated in log-phase growth at a concentration of 5×10^5 cells/ml for 96 hr. FL5.x_L cells treated with paclitaxel or topotecan were treated for 72 hr, and the primary embryonic fibroblast cells were treated at 50% confluency for 48 hr.

Northern Blotting

RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Ten-microgram samples of RNA (quantitated by spectrophotometry) from untreated and treated cells were run on 1% agarose and blotted onto nitrocellulose. Blots were hybridized with ³²P-labeled probes derived by nick translation (Invitrogen) of DNA fragments over 200 bp or by polynucleotide kinase end-labeling (Promega, Madison, WI) of oligonucleotide probes. Oligonucleotide probes included a probe specific for the 18S rRNA (5'-CGC-GGTTCTATTTTGTGTT-3'). Ethidium bromide staining of the agarose gels was performed to confirm equal RNA loading as assessed by staining of the 28S and 18S rRNAs.

Nuclear Run-on

Blot preparation—A slot-blot was created using a HyBond membrane (Amersham Biosciences, Piscataway, NJ) with 5 μ g of B2, λ -phage, and 18S DNA immobilized onto the membrane. Three separate but identical blots were made for each nuclear run-on experiment, one to hybridize with RNA isolated from each treatment group (untreated, vincristine, and etoposide). Plasmids constructed with cloned segments of B2 and 18S DNA were used as the source for DNA. B2 and 18S were amplified from genomic DNA using the following primers: B2-F (5'-CGA-CTGTAAAAGCACTGACTG-3'), B2-R (5'-CTT-CATATATGTCAGTACACCATCAC-3'), 18S-F (5'-CCTGAGAAACGGCTACCACATC-3'), and 18S-R (5'-CATCTAAGGGCATCACAGACCTG-3'). Amplified DNA was cloned into pCR2.1 (creating B2:TA and 18S:TA plasmids) (Invitrogen), maxiprep (Qiagen, Valencia, CA) and used as a source from which all slot-blot DNA was used. Each time a slot-blot was prepared, the B2:TA or 18S:TA vectors were digested with *Eco*RI, and the insert band was gel purified using the QIAquick Gel Extraction Kit (Qiagen). Concentrations of purified DNA were determined using a high DNA mass ladder (Invitrogen). The Lambda-*Hind*III ladder was used as the λ -phage DNA source (negative control for hybridization). All samples were brought to equal volumes using the Qiagen elution buffer from the gel extraction kit. Ethidium bromide was added to each sample (0.2 μ l of a 1 mg/ml stock) to allow for visualization of the DNA on the membrane. DNA was denatured for 10 min at 95°C in an equal volume of 0.8 M NaOH/20 mM EDTA denaturing solution. The reaction was neutralized by the addition of an equal volume of 6 $\times$ SSC, followed by a 10-min room temperature incubation. DNA was vacuum-fixed to the membrane using a Bio-Dot

apparatus, following the manufacturer's protocol (Bio-Rad, Hercules, CA). Following the addition of the DNA, 500 μ l of 0.5 M NaOH was vacuum-drawn through each slot. DNA was UV-crosslinked to the membrane, and the membranes were stored at -20°C between two sheets of filter paper soaked in $2\times$ SSC until ready for use.

Nuclear isolation— 2×10^7 Cells were treated at a density of 5×10^5 cells/ml in media containing 10 $\mu\text{g/ml}$ etoposide or 0.1 $\mu\text{g/ml}$ vincristine for 96 hr, or left untreated. Following cytotoxic treatment, live cells were separated on a glucose-gradient using Lympholyte-M (CedarLane Laboratories, Burlington, NC). From each treatment group, 5×10^6 live cells were used for the subsequent nuclear isolation. Cells were washed twice with ice cold PBS and transferred to a 15-ml conical tube. Cells were pelleted by centrifugation at 500g at 4°C . Following aspiration of the supernatant, the cell pellet was vortexed for 5 sec, followed by the addition of 8 ml NP-40 lysis buffer (buffer solution components listed later) while continuing to vortex the solution for another 10 sec. Lysed cells were incubated on ice for 5 min, followed by a 5-min centrifugation at 500g at 4°C . Following aspiration of the supernatant, the lysis step was repeated again.

In vitro transcription—Each cell pellet obtained by the earlier method was resuspended in 20 μ l of in vitro transcription buffer. To each reaction, 30 μ l of the radioactive in vitro transcription component solution was added to each tube, followed by gentle mixing. Glycerol was added dropwise to the top of the solution, and in vitro transcription was allowed to occur for 60 min at 30°C . Yeast tRNA (4 μ l of 10 $\mu\text{g/ml}$; Ambion, Austin, TX) and Turbo DNaseI (10 μ l of 2 U/ μ l; Ambion) was added to each solution, and incubated for 40 min at 30°C , with an additional 10 μ l of DNaseI added after 20 min. Following this incubation, 120 μ l of $2\times$ proteinase-K buffer and 5 μ l of 20 mg/ml proteinase-K were added to each reaction, and incubated overnight at 42°C .

RNA isolation—Radioactive in vitro transcribed RNA was isolated using Trizol according to the manufacturer's protocol (Invitrogen) and excess unbound radioactivity was removed through separation on a Sephadex G-50 spin column (Roche, Indianapolis, CA). All of the RNA was added to each respective membrane that had been prepared as described later.

Prehybridization, hybridization, and development—After each membrane had thawed at room temperature for at least 30 min, they were prehybridized in ExpressHyb solution for 30 min at 60°C (Clontech, Mountain View, CA), followed by the addition of the radioactive RNA probes (previously isolated in vitro transcribed RNA). Blots were incubated with the radioactive RNA probes for 2 hr at 60°C , followed by two 15-min washes in $2\times$ SSC/ 0.1% SDS at room temperature, and two washes in $0.1\times$ SSC/0.1% SDS for 10 min at 50°C . Each blot received exactly the same volume of wash buffer, as well as duration of wash. Membranes were exposed to film at -80°C .

Buffer solutions—All buffer solutions were made using DEPC-treated H_2O . NP-40 lysis buffer [10 mM TrisCl (pH 7.4), 10 mM NaCl, 3 mM MgCl_2 , and 0.5% NP-40; autoclaved], in vitro transcription buffer [0.3 M $(\text{NH}_4)_2\text{SO}_4$, 4 mM MgCl_2 , 0.2 M NaCl, 0.4 mM EDTA, 4 mM MnCl_2 , 0.1 M TrisCl (pH 7.9), 0.1 mM PMSF, and 1.2 mM DTT], in vitro transcription component solution [per each reaction: 1 μ l each of 10 mM riboGTP, ATP, and CTP (Ambion), 5 μ l of 20 U/ μ l SUPERase-In (Ambion), 10 mM phosphocreatine, 2.5 μ l of 10 mCi/ml [α - ^{32}P]UTP, DEPC-treated H_2O to 30 μ l], and $2\times$ proteinase-K buffer [40 mM TrisCl (pH 7.5), 2% (w/v) SDS, and 20 mM EDTA (pH 8.0)].

Real Time PCR

RNA was isolated using Trizol from variously treated cells, and incubated with DNase I (Invitrogen) according to manufacturer's instructions to remove any contaminating DNA. First strand cDNA synthesis was performed using a random hexamer primer and SuperScript II reverse transcriptase (Invitrogen), according to manufacturer's instructions. One microliter of the resulting cDNA was used in each real time PCR reaction. The SYBR[®] Green PCR Master Mix (Applied Biosystems, Foster City, CA) was used to detect PCR products. PCR reactions were prepared following manufacturer's provided instructions. PCR primers were designed to amplify murine B2 (left primer: 5'-ATGAAAGGAGGCTGGTGAGA-3'; right primer: 5'-AGCTGTCTTCAGACACTCCAGA-3') and murine 18S rRNA (left primer: 5'-CGCGTTCT-ATTTTGTTGTT-3'; right primer: 5'-AGTCGG-CATCGTTTATGGTC-3'). PCR cycle conditions were: 50°C for 2 min, 95°C for 10 min, 95°C for 15 sec, 60°C for 1 min, with the last two steps repeated for 40 cycles. Data were normalized to 18S rRNA as an internal control, and levels of transcription were determined using a known standard curve for each gene. Averages were obtained from three separate amplifications, and error bars represent the standard error of the mean (SEM).

RNA Stability

FL5.x_L cells were treated with 10 µg/ml etoposide for 96 hr. Eight hours before the end of etoposide treatment, 10 µg/ml actinomycin-D (Sigma, St. Louis, MO) was added to the media. Every 2 hr up until the end of the 96-hr etoposide treatment, actinomycin-D was added to a separate cell culture flask, yielding five separate flasks containing cells that were treated with etoposide, as well as actinomycin-D for 0, 2, 4, 6, and 8 hr. Additionally, as a vehicle-only control, side-by-side flasks were treated with an equal volume of ethanol. Finally, duplicate flasks for all of the previously listed treatments were created using cells that had not been treated with etoposide. Therefore, the following cell treatment combinations were made: untreated with actinomycin-D or ethanol added at 0, 2, 4, 6, and 8 hr; etoposide treated with actinomycin-D or ethanol added at 0, 2, 4, 6, and 8 hr. Following all cell treatments, RNA was isolated using Trizol according to manufacturer's instructions (Invitrogen). This RNA was prepared and analyzed for levels of B2 transcription in semi-quantitative real time PCR as described earlier. Specifically, B2 expression was normalized first to levels of 18S rRNA and then expressed as a fold decrease in expression when compared with cells that had not received actinomycin-D or ethanol within the etoposide or untreated group.

To determine statistical significance of the rate of B2 RNA degradation in untreated versus etoposide-treated cells, linear regression models with log scale B2 as the response were used to test for significant associations with time and treatment. First, a model was fit using a quadratic term for time as a continuous covariate. A larger model was then fit that added terms for treatment and treatment by time interactions. A nested *F*-test was used to assess whether these additional main effects and interaction terms were significant, which would suggest that the change in B2 over time differed between treatment groups.

Comet Assay

FL5.x_L cells were treated at 5×10^5 cells/ml with 10 µg/ml etoposide or 100 µM merbarone for 24 hr. Alternatively, cells were treated at 1×10^5 cells/ml with 100 µM H₂O₂ for 20 min on ice. Following treatment, cells were washed with PBS, and the neutral conditions for the CometAssay (Trevigen, Gaithersburg, MD) were performed as per the manufacturer's protocol. Immobilized SYBR Green-stained cells were visualized using the FITC filter on a fluorescent microscope. Comet tail length and tail moment were quantified using a public domain image-analysis program (Helma and Uhl, 2000). Using this program, at least 100

cells/treatment group were analyzed, and average values for tail length and tail moment were determined for each treatment group.

RESULTS

Genotoxic Agents Upregulate Intracellular SINE Transcript Levels

Exposure to genotoxic agents is associated with induction of apoptosis. To characterize cellular responses to genotoxic stress without the confounding issue of cell death, we initially characterized SINE transcript levels in a murine cell line (FL5.12) transfected with a construct driving over-expression of the anti-apoptotic protein Bcl-x_L (FL5.x_L cells; Rudin and Thompson, 2001). We subsequently demonstrated that similar upregulation of SINE transcript levels could be induced by genotoxic exposure in multiple murine and human cell lines.

To further define the spectrum of genotoxic agents that induce upregulation of SINE transcript levels, we evaluated FL5.x_L cells treated with representative members of a broad spectrum of cytotoxic agents (Table 1). Doses and durations of exposure were chosen based on similar cytotoxic efficacy. We previously demonstrated that etoposide, a topoisomerase II inhibitor, was particularly potent in upregulating SINE transcript levels, while vincristine, a microtubule inhibitor without direct DNA-damaging activity, did not induce any significant change in SINE transcript levels (Rudin and Thompson, 2001; Hagan et al., 2003). To evaluate whether the effect of etoposide applied to a broader class of topoisomerase inhibitors, we treated cells with a similarly cytotoxic exposure of topotecan, a topoisomerase I inhibitor. Treatment with this agent was associated with marked upregulation of SINE transcript levels (Fig. 1). To evaluate whether the inability of vincristine to induce SINE transcription in this cell line was representative of microtubule agents as a class, we also treated cells with a similarly cytotoxic exposure to paclitaxel. Vincristine prevents microtubule assembly while paclitaxel inhibits microtubule disassembly; both agents interfere with chromosome separation and promote a G2/M phase arrest. Like vincristine, paclitaxel exposure did not result in any evident change in SINE transcript level (Fig. 1). FL5.12 cells are dependent on the growth factor IL-3 for survival and proliferation; IL-3 withdrawal results in inhibition of multiple intracellular signaling pathways regulating cell growth, proliferation, and survival, resulting in G1 cell cycle arrest and, in the absence of Bcl-x_L, apoptotic induction. As shown in Figure 1, growth factor withdrawal in these cells is not associated with SINE transcriptional induction. Genotoxic agents that induce either single-strand (topotecan) or double-strand (etoposide) DNA breaks can induce upregulation of SINE transcript levels. These data are in agreement with experiments performed previously in our laboratory using other agents that induce either single- (UV) or double-strand (γ -radiation) DNA breaks (Rudin and Thompson, 2001). Conversely, when cells are exposed to cytotoxic agents that are not directly genotoxic (vincristine, paclitaxel, growth factor withdrawal) SINE transcription is not increased. Taken together, these data demonstrate a clear and consistent correlation between genotoxic exposure, but not other similarly cytotoxic exposures, and upregulation of SINE transcript levels (Table 1).

DNA Damage Increases the SINE Transcriptional Rate

At least two mechanisms might account for the increase in SINE transcript levels observed following treatment with DNA-damaging agents: an increase in the actual basal rate of SINE transcription or, conversely, an increase in transcript stability. To separately evaluate each of these two hypothetical mechanisms, we performed nuclear run-on assays to detect changes in the rate of transcription, and RNA stability assays using an RNA polymerase inhibitor to analyze relative transcript stability.

A nuclear run-on assay was performed to determine if etoposide treatment increased the rate of SINE transcription. FL5.x_L cells were treated for 96 hr with etoposide, vincristine, or neither agent. Following the respective treatment, in vitro transcribed ³²P-radiolabeled RNA was collected from isolated nuclei. RNA from each of the three treatment groups were used as probes on membranes containing fixed amounts of SINE DNA (the murine B2 element), 18S DNA (positive control), and λ -phage DNA (negative control) (Fig. 2a). Vincristine exposure was not associated with any evident increase in SINE transcriptional activity relative to untreated cells (Fig. 2a; center). However, a similarly cytotoxic exposure to etoposide led to a 1.6-fold increase in the rate of SINE transcription (Fig. 2a; right). These data indicate that activation of novel SINE transcription is a primary mechanism underlying the increase in SINE transcript levels seen following genotoxic exposure.

To evaluate whether an increase in SINE RNA stability might also contribute to the observed increase in transcript level, we performed RNA stability assays in the presence or absence of etoposide. Actinomycin-D is a well-characterized universal RNA polymerase inhibitor. In the presence of pharmacologic concentrations of actinomycin-D, new RNA transcripts cannot be generated. FL5.x_L cells were treated for 96 hr with or without etoposide, and 10 μ g/ml actinomycin-D was added to aliquots at serial time points between 0 and 8 hr before the end of the 96-hr incubation. RNA was isolated from each aliquot and used as a template in semi-quantitative real-time PCR. In the absence of etoposide, the levels of B2 transcript decreased as a function of time of exposure to actinomycin-D (Fig. 2b). The relative quantity of B2 transcript similarly decreased over time of actinomycin-D treatment in the presence of etoposide; in fact the rate of decline in SINE RNA was significantly greater in cells treated with etoposide than in control cells (Fig. 2b; $P < 0.0001$ for the difference between degradation rates). Taken together, these data demonstrate that the upregulation of SINE transcript level following genotoxic exposure can be attributed to an increase in SINE transcriptional rate, with no evident increase (and in fact a moderate decrease) in transcript stability.

SINE Activation by Topoisomerase Inhibitors Correlates with DNA Cleavage

Multiple commonly used topoisomerase inhibitors, including inhibitors of both topoisomerase I (topotecan) and topoisomerase II (etoposide), were found to be potent inducers of SINE transcription. One hypothesis to explain this association is that increased DNA strand cleavage from exposure to these agents triggers a DNA damage response pathway resulting in SINE activation. Alternatively, topoisomerase inhibition leading to distortion of DNA structure and cell cycle arrest might in some way promote SINE activation in the absence of DNA strand breakage. To distinguish between these alternative hypotheses, we compared the effects of two topoisomerase II inhibitors with distinct mechanisms of action, etoposide and merbarone. Etoposide acts by stabilizing the reaction intermediate generated by double strand cleavage by topoisomerase II, preventing strand religation and resulting in increased double strand breaks. Merbarone is an alternative topoisomerase II inhibitor that acts by inhibiting the initial double strand cleavage by topoisomerase II (Brewer et al., 1985; Cooney et al., 1985; Glover et al., 1987). Both agents inhibit cell cycle progression and DNA replication, and both are similarly cytotoxic.

FL5.x_L cells were treated for 96 hr with similarly cytotoxic doses of etoposide, merbarone, vincristine, or none of these agents, and RNA was extracted for northern blot analysis. As previously demonstrated, etoposide was strongly associated with SINE transcriptional activation (Fig. 3). In contrast, no induction of SINE transcription was observed in cells treated with merbarone. The clear dichotomy in outcome between these two topoisomerase II inhibitors strongly suggested that strand breakage was a key component of SINE activation by topoisomerase inhibitors.

It remained formally possible that merbarone, although not directly DNA-damaging, was promoting torsional strand breakage through inhibition of topoisomerase activity. If this were the case, this would provide a counterexample to the apparently tight association between DNA damage and SINE activation. We used a comet assay to directly compare the extent of chromosomal breakage in cells treated with merbarone and etoposide. This assay is based on immobilizing cells in low-melt agarose and exposing the cells to an electrical current. Following exposure to the current, cells are stained with SYBR Green, a fluorescent nucleic acid stain that allows for visualization of DNA. Fragmented DNA freely migrates out of the nucleus in contrast to intact chromosomal DNA, which remains fixed in the nuclear compartment. The extent of DNA fragmentation can be quantitated through characteristics of the distinctive comet tail.

FL5.x_L cells were treated with 10 µg/ml etoposide, 100 µM H₂O₂ (positive control for DNA damage), 100 µM merbarone, or were left untreated. Cells were treated under neutral comet assay conditions, immobilized, and exposed to the electrical field. Following incubation with SYBR Green, cells were visualized using fluorescence microscopy. Representative images were documented for each treatment group (Fig. 4a). In etoposide- and H₂O₂-treated cells (right side of Fig. 4a), the formation of comet tails was evident, indicative of damaged DNA migrating out of the nucleus. However, in untreated cells or cells treated with merbarone (left side of Fig. 4a), the nuclei and stained DNA remained intact. The extent of DNA fragmentation can be characterized using two quantitative measures: the relative length of the comet tail, and the tail “moment,” a calculation that involves both tail length and the relative staining intensity of the tail (Helma and Uhl, 2000). In cells treated with H₂O₂ or etoposide, both potent inducers of DNA damage, tail length and tail moment increased 3–5-fold over untreated cells (Fig. 4b). Cells treated with merbarone did not show an increase in tail length or moment when compared with untreated cells. Based on this assay, etoposide, but not merbarone, was associated with significant DNA strand breakage. These data offer further confirmatory support for the close association between DNA damage and SINE activation.

Trp53 Inhibits SINE Activation

We have evaluated genotoxic agents representative of a variety of classes and mechanisms of action. Certain downstream cellular responses to DNA damage, including activation of TP53, are shared among many damage response pathways. We were interested in specifically defining the contribution of Trp53 on SINE activation following treatment with DNA-damaging agents. To address this question, we evaluated stable transfectants of FL5.x_L expressing a dominant-negative Trp53 construct. These cells, FL5.x_L.DD, lack functional Trp53, and when exposed to DNA-damaging agents will not arrest at normal cell cycle checkpoints (Minn et al., 1996). FL5.x_L.DD cells were treated for 96 hr with etoposide, vincristine, or neither of these agents. RNA isolated following these treatments was analyzed by northern blot to determine levels of SINE transcription (Fig. 5a). Surprisingly, the SINE transcriptional response following treatment with DNA-damaging agents was markedly augmented in cells lacking functional Trp53 relative to control cells with intact Trp53. Confirmation of these data by semiquantitative real time PCR demonstrated a 27-fold increase in SINE transcription following treatment with etoposide in FL5.x_L.DD cells (Fig. 5b), ~4-fold higher levels of transcriptional induction than that observed in FL5.x_L cells under similar conditions. This data suggests that functional Trp53 represses the DNA damage-induced SINE transcription response.

Recent data suggest that mutant *TP53* alleles, previously thought to act primarily as loss-of-function alleles, can in fact manifest dominant-negative effects that are distinct from genetic loss of *TP53*. To evaluate whether the hyperresponsiveness of DNA damage-induced SINE transcription in cells expressing dominant-negative Trp53 was in fact attributable to Trp53

inactivation, as opposed to an aberrant activity of the dominant-negative allele, we also examined DNA damage-induced SINE transcription in syngeneic mouse primary embryonic fibroblasts containing heterozygous or homozygous *Trp53* gene deletions (Donehower et al., 1992; Frank et al., 2000). RNA was isolated from both cell lines following 48 hr in the presence or absence of etoposide. These lines, not transfected with Bcl-x_L, were treated with a shorter time course than that used previously for FL5.x_L cells to avoid excessive cell death that would confound analysis of expression data. Northern analysis using RNA isolated from the *Trp53* heterozygous and homozygous deletants concurs with the data obtained in FL5.x_L.DD cells (Fig. 5c). SINE transcription following treatment with etoposide was markedly higher in *Trp53* null cells than in matched control cells expressing wild type Trp53. Baseline levels of SINE transcription, even in the absence of exogenous treatment, were also somewhat higher in cells lacking functional Trp53 than in controls. These data indicate that Trp53 has a modest suppressive effect on SINE transcription at baseline, and a major effect in attenuating SINE transcriptional activation following treatment with DNA-damaging agents.

DISCUSSION

We demonstrate that there is a consistent association between exposure to DNA-damaging agents and upregulation of SINE transcripts, that this upregulation is specifically attributable to transcriptional activation, and that this transcriptional activation is attenuated by Trp53. The association between genotoxic exposure and induction of SINE transcription is remarkably robust. We consistently observe SINE transcriptional upregulation following treatment with a wide variety of DNA-damaging agents. These agents include representatives of disparate therapeutic classes: differing in mechanisms of action, in the types of resulting damage, and in the predominant repair pathways induced. Activating agents include drugs that cause inter-strand and intrastrand crosslinks, DNA adduct formation, and single and double strand DNA breaks. Analysis of SINE transcriptional induction following treatment with distinct classes of topoisomerase inhibitors has been particularly informative. Clinically relevant topoisomerase I and topoisomerase II inhibitors, associated with stabilization of single strand and double strand cleavage intermediates, respectively, are potent inducers of SINE transcription. In contrast, we found that exposure to the atypical topoisomerase II inhibitor merbarone, associated with inhibition of DNA cleavage rather than with stabilization of the cleavage intermediate, has no evident ability to induce SINE transcription. The clear difference between effects of similarly cytotoxic exposures to etoposide and merbarone, two agents targeting the same regulator of DNA topology, strongly supports the association between DNA damage, strand breakage, and SINE activation.

The association between strand breakage and SINE transcription may be due to secondary changes in chromatin architecture following exposure to DNA-damaging agents such as etoposide and topotecan. Alu elements tend to be located in areas of dense heterochromatin. Chromatin structure surrounding SINE elements may play a key role in sequestering these elements to transcriptionally silent regions of the genome (Ullu and Weiner, 1985; Carey et al., 1986; Russanova et al., 1995). Available evidence suggests that murine SINE elements are particularly subject to this type of transcriptional control through interactions with chromatin proteins (Carey et al., 1986). Moreover, some treatments (adenoviral infection, heat shock) that have previously been shown to induce SINE transcription also modulate chromatin structure (Russanova et al., 1995; Kim et al., 2001). The activation of SINE transcription following treatment with DNA-damaging agents described in this study may be dependent on the same mechanism. Strand breakage due to the mechanistic action of the DNA-damaging agent may lead to changes in chromatin architecture with secondary

upregulation of SINE transcription due to the release of higher order epigenetic transcriptional repression.

The mechanisms by which disparate DNA-damaging events are recognized and trigger SINE transcriptional activity are a focus of ongoing investigation. In contrast to most mRNA of the cell, transcription of SINE elements is mediated by RNA polymerase III (pol III). The cellular responses to DNA damage do not include a generalized upregulation of polymerase III activity; none of several other pol III-dependent genes we have evaluated appear responsive to any class of genotoxic agent. Thus this transcriptional activation appears to be relatively specific to SINEs.

No evidence of an increase in SINE RNA message stability was observed following genotoxic exposure; in fact, the converse observation of a slight increase in the rate of SINE mRNA degradation was noted in etoposide-treated cells. Therefore it would appear that the marked upregulation of SINE mRNA in these cells can only be attributed to an increase in transcriptional rate. Only a modest increase in transcriptional rate, 1.6-fold, was evident by nuclear run-on. Because SINE transcripts are commonly embedded in polymerase II (pol II) transcripts, an expected high background of pol II transcription in all treatment groups using a nuclear run-on assay may mask a greater increase in true de novo SINE transcripts; the fractional increase detected by nuclear run-on analysis may underestimate the actual change in SINE transcriptional rate. Consistent with this hypothesis, the observed increase by northern blot is much greater than that detectable by nuclear run-on.

Upregulation of TP53 activity, primarily by protein stabilization, is a central cellular response to genotoxic stress induced by multiple forms of DNA damage (Chowdary et al., 1994; Maki et al., 1996). We therefore sought to evaluate whether Trp53 might be an important component of the signaling pathway leading from generalized DNA damage to SINE transcriptional induction. Our data do not support this hypothesis and in fact confirm the converse: functional Trp53 attenuates the activation of SINE transcription following genotoxic exposure. This result was confirmed both through expression of a dominant-negative form of Trp53 that abrogates Trp53-dependent cell cycle checkpoint responses, and through parallel evaluation of matched cell lines with and without *Trp53* homozygous gene deletion.

In addition to the clear suppressive effect on DNA damage-triggered SINE transcription, our studies demonstrate that functional Trp53 has a moderate suppressive effect on SINE transcription at baseline. This relatively minor basal effect on SINE transcription has been previously noted by others (Chesnokov et al., 1996). Chesnokov et al. suggest that the N-terminal transactivation domain of TP53 can repress Alu element transcription in a manner that is dependent on the internal pol III promoter; expression of Alu elements juxtaposed to strong genomic pol III promoters was not TP53-responsive. These authors suggest that this effect may be mediated by an inhibitory association between TP53 and the polymerase III transcription factor, TFIIB. The authors speculate that this association between TFIIB and TP53 represses SINE transcription by depleting an essential transcription factor. Our data are consistent with this model. Alu elements may be more susceptible to disruption of the transcriptional machinery (i.e., depletion of TFIIB) than other pol III transcribed genes due to their relatively weak and poorly conserved pol III promoters. This may be why transcription of Alu elements is specifically affected by TP53 loss, whereas other pol III transcribed genes remain largely unaffected. The role(s) of upstream DNA damage sensors that lead to TP53 activation (ATM, ATR, BRCA1, etc.) in mediating these effects has not been defined.

The observed suppressive effect of Trp53 on SINE activation following DNA damage represents yet another dimension of the ability of TP53 to function as a global protector of genomic integrity. SINE elements are by far the most common retrotransposable element, present in over one million copies per human genome (Lander et al., 2001). Absence of wild type TP53 function in cancer cells is thought to contribute to the heterogeneity of cancer cells due to loss of critical cell cycle checkpoint responses, loss of damage-mediated apoptotic induction, and failure to activate DNA damage repair pathways. Failure to suppress activation of retrotransposable genetic elements following treatment with commonly used genotoxic anticancer therapies may further promote chromosomal alterations in cancer cells lacking functional TP53 response pathways. Experiments to determine the genomic impact of the association between TP53 inactivation and DNA damage mediated activation of mobile genetic elements in malignant cells will provide interesting insights into possible secondary effects of genotoxic therapy.

Secondary acute myeloid leukemia (AML) following exposure to topoisomerase II inhibitors, including etoposide, frequently involve translocations of the *MLL* gene at 11q23 (Albain et al., 1990; Aplan et al., 1996; Broeker et al., 1996). Although *MLL* translocations are frequently observed in primary as well as secondary AML, the translocation breakpoints of secondary AML tend to cluster within a distinct region of the gene (Broeker et al., 1996).

Exposure to etoposide and similar topoisomerase II inhibitors has been clearly linked to secondary AML containing *MLL* gene translocation. Of all of the potential topoisomerase cleavage sites within the genome, it is unclear why the *MLL* locus would be preferentially involved in secondary malignancies. One explanation may be increased accessibility of this chromosomal region to topoisomerase II or the topoisomerase II/etoposide complex. Increased transcriptional activity can markedly increase accessibility of a chromosomal site to endonucleolytic cleavage. The *MLL* locus is notably Alu-rich, with eight Alu elements within the 8.3-kb breakpoint cluster region alone (Gu et al., 1994). It is conceivable that the increased transcriptional activity of these specific Alu elements in response to etoposide exposure serves to increase accessibility of this region for breakage and translocation in the presence of topoisomerase II and a topoisomerase II inhibitor.

Given the extremely high prevalence of these mobile elements within the genome, even low-level activation of SINEs by endogenous or exogenous mutagens might contribute significantly to chromosomal rearrangement. The high prevalence of Alu elements within the breakpoint region associated with secondary AML suggests a potential role in aberrant chromosomal rearrangements seen after genotoxic exposure. Developing a more complete understanding of the cellular responses to high dose genotoxic therapy may yield insights into the etiology of secondary malignancies, may be useful in predicting the carcinogenic potential of those therapies, and may assist in the development of anticancer therapies with lower long-term leukemogenic risk.

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Figure 1.

DNA-damaging agents activate SINE transcription. FL5.x_L cells were treated at a density of 5×10^5 cells/ml with 0.1 μ g/ml vincristine, 10 μ g/ml etoposide, 5 μ g/ml paclitaxel, or 1 μ M topotecan. Cells were treated for 96 hr, with the exception of paclitaxel and topotecan with which cells were treated for 72 hr. Cells that were withdrawn from growth factor (IL3) were washed repeatedly with PBS, and grown in media without IL3 for 18 hr. RNA isolated from the variously treated cells was analyzed by northern blot analysis. The blot was sequentially hybridized with radioactive probes complimentary to the B2 SINE element and 18S rRNA (loading control).

Figure 2.

DNA-damaging agents increase the rate of SINE transcription. (a) Nuclear run-on assay to determine changes in the rate of SINE transcription. Radioactive in vitro transcribed RNA from variously treated cells was analyzed using hybridization to fixed amounts of DNA. Relative levels of B2 expression were first compensated to 18S levels, and then expressed as a fold increase over untreated cells. These values represent an average obtained from three separate experiments, with SEM listed below each value. (b) Analysis of RNA stability following treatment with etoposide. Cells were treated for 96 hr with etoposide, or left untreated. In the last 0–8 hr of etoposide or sham treatment, 10 μ g/ml actinomycin-D (ACD) was added every 2 hr. Isolated RNA was used in semiquantitative real-time PCR to analyze stability of B2 transcripts over time in the presence of ACD. Data are graphed as a function of time comparing the rates of B2 degradation in untreated and etoposide-treated cells. The curves of log scale B2 expression versus time of exposure to ACD were significantly different, with the etoposide-treated group exhibiting a more rapid rate of decline ($P < 0.0001$). Error bars: SEM from triplicate experiments.

Figure 3.

SINE transcription correlates with DNA cleavage. FL5.x_L cells were treated at a density of 5×10^5 cells/ml with 0.1 μ g/ml vincristine, 10 μ g/ml etoposide, or 100 μ M merbarone for 96 hr. Following treatment, RNA was isolated and analyzed by northern blot analysis. The blot was sequentially hybridized with radioactive probes complimentary to the B2 SINE element and 18S rRNA (loading control).

Figure 4.

Merbarone does not induce DNA breaks. (a) Comet assay images. Cells were treated with 10 μ g/ml etoposide, 100 μ M merbarone, 100 μ M H₂O₂, or left untreated. Cells were analyzed under neutral comet assay conditions using SYBR Green to visualize nuclei and comet tails. Fluorescent images were obtained using the FITC filter. Representative images from each treatment group are shown at 10 $\times$ magnification. (b) Quantification of comet assay data. Using a public domain image-analysis program, tail length and tail moment were measured for cells that were untreated, or treated with H₂O₂, etoposide, or merbarone. Data are represented as an increase in tail length and tail moment when compared with untreated cells. Data represent an average of values determined from at least 100 cells/ treatment group from three separate experiments. Error bars represent SEM.

Figure 5.

p53 represses SINE transcription. (a) SINE transcription is augmented in cells expressing a dominant-negative p53. FL5.x_L or FL5.x_L.DD cells were treated at a density of 5×10^5 cells/ml for 96 hr with 0.1 μ g/ml vincristine, 10 μ g/ml etoposide, or left untreated. RNA was isolated following treatment and analyzed by northern blot analysis. The blot was hybridized with a radioactive probe complementary to the B2 SINE element. Ethidium bromide staining is shown to indicate equal loading in all lanes. (b) Quantification of SINE transcription in cells expressing dominant-negative p53. RNA isolated from FL5.x_L and FL5.x_L.DD cells treated as described in (a) was used to determine levels of B2 expression using semiquantitative real time PCR. Relative levels of B2 expression are shown as a fold increase over untreated cells within each cell line. Error bars represent SEM. (c) SINE transcription in p53 knockout fibroblasts. p53 +/- heterozygous and p53 -/- homozygous primary mouse embryonic fibroblast cells were treated at 50% confluency with 10 μ g/ml etoposide for 48 hr or left untreated. RNA isolated from these cells was analyzed using

northern analysis. The blot was sequentially hybridized with probes complimentary to B2 and 18S rRNA.

TABLE 1**Cytotoxic Therapies Evaluated for SINE Transcriptional Induction**

Agent	Class	Primary DNA lesions	SINE induction	Reference
Cisplatin	Crosslinking agent	Intra-/inter-strand crosslinks, single/ double strand breaks	Yes	Rudin and Thompson, 2001
Etoposide	Topo II inhibitor	Double strand breaks	Yes	Figures 1, 3, and 5
Topotecan	Topo I inhibitor	Single strand breaks	Yes	Figure 1
γ -radiation	Ionizing radiation	Double strand breaks	Yes	Rudin and Thompson, 2001
UV-radiation	Nonionizing radiation	Pyrimidine dimers, single strand breaks	Yes	Rudin and Thompson, 2001
IL-3 withdrawal	Apoptotic trigger	None	No	Figure 1
Merbarone	Topo II inhibitor	None	No	Figure 3
Paclitaxel	Microtubule inhibitor (blocks disassembly)	None	No	Figure 1
Vincristine	Microtubule inhibitor (blocks assembly)	None	No	Figures 1, 3, and 5