Methodological Report

In Situ Detection of Specific DNA Double Strand Breaks Using Rolling Circle Amplification

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KEY WORDS

DNA, double strand break, rolling circle amplification, HO endonuclease, adenovirus

ABBREVIATIONS

- DSB double strand breaks
- RCA rolling circle amplification
- IF immunofluorescence
- PIKK phosphatidylinositol 3-kinase like kinase
- ChIP chromatin immunoprecipitation
- SSB single-stranded DNA binding protein m.o.i. multiplicity of infection

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ABSTRACT

We have developed a method to localize DNA double strand breaks (DSBs) in situ in cultured mammalian cells. Adenoviruses encoding *Saccharomyces cerevisiae* HO endonuclease and its cleavage site were used to induce site-specific DSBs. Rolling circle amplification (RCA), a sensitive method that allows the detection of single molecular event by rapid isothermal amplification, was used to localize the broken ends in situ. Punctate RCA signals were only seen in the cells that had been infected with both adenoviruses encoding HO endonuclease and HO cleavage site, but not in the cells mock-infected or infected with the site or endonuclease virus only. With use of a chemical crosslinker, in situ RCA and immunofluorescence (IF) can be performed simultaneously on the same sample. This methodology provides a novel approach for investigation of DNA recombination, DNA repair, and checkpoint controls in mammalian cells.

INTRODUCTION

DNA damage activates checkpoint pathways that arrest the cell cycle, induce transcription of damage-response genes, and/or cause apoptosis. DNA damage checkpoint pathways, which are well-conserved from yeast to mammals, are vital for humans, as genetic instability arising from checkpoint failures contributes to diseases including cancer. DNA damage checkpoint pathways are akin to other signal transduction pathways, in which a signal (generated by DNA damage) is detected and transduced to activate downstream amplification and effector pathways. In mammals, there is much information about the amplification and effector pathways, but knowledge about how damaged DNA activates damage response proteins is more limited. Rad17 (a RFC-like protein) and PCNA-like proteins Rad9, Rad1 and Hus1 are among the damage sensor candidates. ATM and ATR, members of the phosphatidylinositol 3-kinase like kinase (PIKK) family, play central roles in damage responses and initial phosphorylation events. ATM is activated mainly by DSBs, while ATR responds to UV-induced damage, DSBs and stalled replication forks. ATM and ATR phosphorylate a broad spectrum of substrates, including Chk1 and Chk2, which are important downstream effector kinases.¹ Exposure to ionizing radiation (IR), which induces various DNA lesions including DSBs, results in accumulation of proteins including γ-H2AX,² ATR,³ and members of the MRN (Mre11-Rad50-NBS1) complex⁴ at discrete foci that are located at sites of DNA damage and repair. Improved methods for precisely localizing DNA damage sites in intact cells in situ, in combination with a parallel protein detection technique, such as IF, would facilitate investigation of DNA damage-activated signaling pathways, especially the putative damage sensors.

Various methods have been adopted for induction of DNA DSBs, an important form of DNA damage to cells, and subsequent detection of the sites of DNA damage. These include physical methods, such as IR and X-rays, as well as enzymatic methods, such as induction of bacterial or yeast endonucleases.⁵⁻⁸ The methods for detecting the DSBs vary depending on how the damage is induced. When the sequences of the DNA broken ends are unknown, for instance, after X-ray treatment, DSBs can be end-labeled with the incorporation of bromo deoxyuridine (BrdU) by terminal deoxynucleotidyltransferase (TdT) and the BrdU is then visualized with fluorophore-conjugated antibody that recognizes BrdU.⁷ When DSBs are induced at designated target sites by endonuclease digestion, Southern blot analysis can be used to confirm the breaks, and chromatin immunoprecipitation (ChIP) can be used to study the proteins associated with the DNA breaks.^{5,6} However, in situ BrdU end-labeling does not exclusively identify the induced DSBs. ChIP, although a powerful method for studying proteins directly or indirectly associated with chromatin, does not have the specificity for broken chromatin, since it produces signals whether proteins are in close proximity to DNA breaks or to intact DNA.

We describe here the use of RCA, an effective signal amplification method, for detection of DSBs induced by HO endonuclease. In situ RCA is a surface-anchored isothermal nucleic-acid amplification reactions based on a rolling-circle replication mechanism. RCA is highly sensitive, and allows the detection of DNA or RNA signals by rapid amplification of the target or a small circularized oligonucleotide probe.9 In the presence of a single primer, hundreds of copies of tandemly linked circle sequence can be generated by DNA polymerase within a few minutes. RCA products can be visualized directly by incorporation of haptens or fluorophores, or indirectly by annealing to fluorophore-labeled decorator oligonucleotides complementary to the RCA product. RCA provides sufficient sensitivity and specificity to detect single-copy genes in human genomic DNA. The method has been used to discriminate alleles and detect point mutations in cultured cells, as well as to detect and quantify mRNA.9-11

An important experimental tool for investigating DSB repair systems was developed in budding yeast. Saccharomyces cerevisiae HO endonuclease normally initiates mating type switching through site-specific cleavage of the mating type (MAT) locus.¹² The engineering of HO endonuclease under an inducible promoter has made it possible to induce DSBs in yeast at designated target sites, as well as in a preferred time frame.^{5,6} This has led to important contributions in the investigation of DSB repair processes and DNA damage checkpoint pathways. We have endeavored to produce a similar system that can be used in mammalian cells. The strategy is to combine an adenovirus/HO endonuclease expression/target system that was previously developed, with in situ RCA. This has enabled us to localize site-directed DSBs in intact cultured mammalian cells. Eventually, it should be possible to adapt this approach for investigation of DNA recombination, DNA repair, and checkpoint controls in mammalian cells.

MATERIALS AND METHODS

DNA oligonucleotides. DNA oligonucleotides were synthesized by the Yale University Department of Pathology DNA Synthesis Lab. The 81 nucleotide open circle probe has the sequence: phosphate-5'-AAACCCTGGTTTTGTATATGATG-GTACCGCAGCCAGCATCACCAGACTGAGTATCTCCC-TATCACTAACAGTATAATTTTAT-3'. The phosphorylated oligonucleotide for ligation and RCA in solution was 5'-CAAAACCAGGGTTTATAAAATTATACTGTT-3'. Decorator oligonucleotides have the same sequence as the backbone of the open circle. Their sequences were: 5'-Cy3-TATATGATGGTACCGCAGCAGCAGCAGCAGCAGCAGCAGdin torma circular l can serv strand-di circle cc amplifica oligonuc

TATATGATGGTACCGCAG-dinitrophenol (DNP)-3' and 5'-Cy3-TGAC TATCTCCTATCACT-DNP-3'.

Cell lines and enzymes. HEK293 and 293T cells were maintained in DMEM supplemented with 10% fetal bovine serum, L-glutamine and antibiotics. T4 DNA ligase was purchased from New England Biolabs. Sequenase version 2.0 DNA polymerase and single-stranded DNA binding protein (SSB) were from USB.

Adenovirus vectors. The construction of virus Ad E1::HO Gene has been described in Nicolás et al.¹³ Virus Ad E1::HO Site B was constructed by first



Figure 1. RCA specifically localizes cleaved HO site. After infecting cells with Ad E1::HO Site B (and Ad E1::HO Gene), cellular DNA is denatured. An 81-nt "open circle" oligonucleotide with ends complementary to 30-nt contiguous sequence within the HO site is then added. Annealing of this oligonucleotide to the denatured HO site will result in formation of a noncovalently closed circle that is next sealed with DNA ligase. If the circular DNA is annealed to a cleaved HO site, the 3'OH group of the cleaved HO site can serve as a primer for amplification of the ligated circle template sequence by a strand-displacing DNA polymerase. The DNA polymerase progresses around the ligated circle continously until the circle sequence is amplified hundreds of times. The in situ amplification product is visualized with complementary fluorophore-labeled decorator oligonucleotides. These oligonucleotides bind to the amplified DNA, which spontaneously condenses into a small, spherical object localized at the site of DNA synthesis (A). In (B), if the HO site has not been cleaved, the open circle is annealed to the HO site and ligated, but there is no 3' end in Ad E1::HO Site B DNA proximal enough to the ligated circle to serve as a primer for DNA amplification. Hence, no signal should result.

inserting the 127 nt *Bam*HI to *Sma*I fragment, containing the MATa HO site from pWJ421, into the *Bam*HI and *Eco*RV sites of the adenovirus shuttle vector pACE.¹⁴ Virus was produced by cotransfecting 293 cells with the modified vector DNA and the adenovirus genome-containing plasmid pJM17.¹⁵ Recombination in vivo between the two plasmid DNAs led to the formation of viable virus. Following plaque-purification, the recombinant structure of the genome was confirmed by restriction digestion and DNA sequence analysis of viral DNA. The HO site is oriented in the opposite direction to that in the *Ad E1::HO Site* virus described earlier,¹³ with the



Figure 2. Analysis of ligation and RCA in solution. (A) Analysis by urea-PAGE of open circle ligated with artificial HO site target. Ligations were performed at 37°C for 1 hour in the presence of T4 DNA ligase. The reaction products were resolved by PAGE with 8% acrylamide and 8M urea in 1x TBE and visualized with SYBR-Green II. Each triangle signifies increased amount of loading of the same reaction products. (B) Analysis of RCA products. A portion of the ligation reaction mixture was used to provide the primer and template for RCA. RCA was proceeded in the presence of Sequenase for 15 min at 37°C, before being stopped with alkaline gel loading buffer. RCA was analyzed on a 0.8% alkaline agarose gel and visualized with SYBR-Green II. Negative control ("neg") was RCA reaction in the absence of ligation mixture. A pair of unrelated circle and target with known high efficient RCA was used as positive control ("pos"). Each triangle signifies increased amount of loading of the same reaction products.



single strand extension TGTT 3' ending at nt 1012 from the left hand end of the viral genome.

Plasmids. For transfection in 293T cells, a plasmid carrying the HO site was constructed by inserting a 141nt BamHI to SalI fragment containing the HO site from the shuttle vector pACE into the BamHI and XhoI sites of pcDNA3.1/V5-HisA (Invitrogen). For construction of plasmid with HO endonuclease coding sequences, intracellular viral DNA was purified from 293 cells infected with adenovirus *Ad E1::HO gene* by a modification of the Hirt technique.¹⁶ The HO endonuclease coding sequence of *Ad E1::HO gene* viral DNA was amplified by PCR and subcloned into pcDNA vector (Invitrogen). The resulting constructs were designated pcDNA-HOsite and pcDNA-HOendo, respectively. All plasmid constructs were verified by sequence analysis.

Ligation and RCA in solution. Ligation reactions were performed in 1x T4 DNA ligase buffer [(50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, 25 μ g/ml bovine serum albumin (BSA)] containing 60 mM NaCl, 0.1% Tween 20 and 125 μ g/ml total BSA with the final concentrations of open circle and target oligonucleotides at 1 μ M and a total volume 50 μ l. Components of the reaction mixture, except for BSA and DNA ligase, were combined, heated at 65°C for two minutes, and cooled to room temperature. After addition of BSA and 5 U/ μ l of T4 DNA ligase, the mixture was incubated at 37°C for 1 hour. The reaction products were resolved by urea-polyacrylamide gel electrophoresis (PAGE) with 8% acrylamide and 8 M urea in 1x Tris-Borate EDTA (TBE) and visualized with SYBR-Green II (Molecular Probes).

A portion of the ligation reaction mixture (2.5 μ l out of 50 μ l) was used to provide the primer and template for RCA, with a total volume of 25 μ l. RCA was carried out in 1x RCA buffer (40 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 50 mM NaCl, 6.7 mM DTT, 0.01% Triton X-100). BSA was added to achieve total concentration of 200 μ g/ml. After addition of 250 μ M dNTPs, the solution was incubated at 42°C for 15 min, then cooled to room temperature. Sequenase (0.6 U/ μ l) and SSB (1.4 μ M) were added and RCA proceeded for 15 min at 37°C, before being stopped with alkaline gel loading buffer (30 mM NaOH, 4 mM EDTA, 12% FicoII and 0.025% bromocresol green). RCA was analyzed on an 0.8% alkaline agarose gel prepared in 1 M NaCl/50 mM EDTA with alkaline electrophoresis buffer (1.5 M NaOH, 50 mM EDTA) and visualized with SYBR-Green II.

In situ ligation and RCA after adenovirus infection. HEK293 cells were grown on 8-well culture slides (Becton Dickinson) to 80-90% confluence. Cells were infected with *Ad E1::HO Gene* and/or *Ad E1::HO Site B* with m.o.i. (multiplicity of infection) of approximately 0.04 (for experiments in (Figs. 3 and 4) or 5 (for experiments in Fig. 5), or mock infected. Eighteen hours after infection, cells were washed twice in phosphate-buffered saline

> (PBS, calcium, magnesium free), fixed in 4% paraformaldehyde (PFA) in PBS for 15 min at room temperature, and permeabilized in 0.5% Triton X-100 in PBS for 15 min

> Figure 3. In situ RCA after adenovirus infection. HEK293 cells were grown on 8-well culture slides to 80-90% confluence. Cells were infected with Ad E1 .: HO Gene and/or Ad E1::HO Site B with m.o.i. of approximately 0.04. Eighteen hours after infection, cells were washed, fixed in 4% PFA, and permeabilized in 0.5% Triton X-100. After DNA denaturation, on slide ligation was carried out in the presence of T4 DNA ligase and 500 nM open circle oligonucleotides at 37°C for 1 hr. RCA was performed at 37°C for 30 min in RCA solution containing Sequenase. After 30 min incubation in blocking buffer at 37°C, adenovirus-infected cells were identified by IF using rabbit anti-adenovirus late structural protein antiserum. FITC-conjugated anti-rabbit IgG was added to the decoration buffer containing two 500 nM Cy3-labeled decorator oligonucleotides, and the slides were incubated at 37°C for 30 min. Nuclei were stained with DAPI.

at room temperature. After three washes in PBS and two washes in 2x SSC (sodium chloride/sodium citrate), DNA was denatured at 70°C for 2 min in 70% formamide in 2x SSC. Slides were immediately immersed in ice cold 70%, 90% and 100% ethanol for 5 min each, and air dried. On slide ligation of a total volume 50 µl was carried out in 1x T4 DNA ligase buffer containing 5 U/µl T4 DNA ligase, 60 mM NaCl, 0.1% Tween20, 200 μ g/ml total BSA, and 500 nM open circle oligonucleotides at 37°C for 1 hr. The slides were washed once in 0.1% Tween20/0.5xSSC at room temperature for 5 min. RCA was performed at 37°C for 30 min in 35 µl RCA solution containing 1x KGB buffer (25 mM Tris-acetate, pH 7.6, 100 mM K-Glutamate, 10 mM Mg-acetate), 0.2 µg/µl BSA, 0.1% Tween-20, 1.4 µM SSB, 200 µM dNTP, 6.7 mM DTT and 0.6 U/µl Sequenase. After two 5-min washes in 0.1% Tween20/2x SSC at 37°C, 35 µl decoration buffer containing two 500 nM decorator oligonucleotides 5'-end labeled with Cy3, 0.1% Tween20, 1% BSA, 1 mg/ml denatured salmon sperm DNA and 2x SSC were added to the slides. Decoration was performed at 37°C for 30 min. Slides were washed twice in 1% BSA, 0.1% Tween20/2x SSC at 37° C for 5 min each. Nuclei were stained with 0.1 µg/ml DAPI. The slides were mounted with Anti-fade mounting medium (Molecular Probes). Images were captured using a Nikon Microphot-FX microscope equipped with Nikon FX-35A camera.

Transient transfection. 293T cells were mock transfected, or transiently transfected with pcDNA vector, pcDNA-HOsite and/or pcDNA-HOendo at a ratio of 1 μ g of DNA to 2 μ l of FuGene 6 (Roche). Cells were fixed approximately 48 hrs after transfection and in situ RCA was performed.

In situ IF. After ligation, RCA and 30 min incubation in blocking buffer (2% BSA, 0.1% Tween 20/PBS) at 37°C, adenovirus-infected cells were identified by IF using rabbit anti-adenovirus late structural protein antiserum (1:200 diluted in blocking buffer). Slides were washed three times in 0.1% Tween20/PBS for 10 min each. Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Jackson ImmunoResearch, diluted to 15 μ g/ml in blocking buffer) was added to the decoration buffer containing two 500 nM Cy3-labeled decorator oligonucleotides, and the slides were incubated at 37°C for 30 min.

For γ -H2AX IF, the IF steps were carried out prior to RCA, after the cells were fixed and permeabilized. Cells were incubated in blocking buffer for 30min at 37°C. Then, cells were incubated with mouse anti-human γ -H2AX (phospho-Histone H2AX (Ser139) monoclonal antibody (Upstate Biotechnology, 1:1000 diluted in blocking buffer) and FITC-conjugated anti-mouse IgG (Jackson ImmunoResearch) diluted to 7.5 µg/ml in blocking buffer. After slides were washed four times in 0.1% Tween20/PBS for 10 min each, cells were incubated in 10mM EGS (ethylene glycol-bis(succinic acid N-hydroxysuccinimide ester), Sigma) /PBS at 37°C for 45 min. Cells were washed in 2xSSC, then in situ RCA was carried out as before.

RESULTS

Specific localization of cleaved HO site by RCA. HO endonuclease recognizes and cleaves an 18-bp recognition site, leaving 4-base 3'-OH overhangs.^{12,17} Specific requirements must be achieved to produce a RCA system that recognizes HO cleavage products. First, the template molecule for RCA is a single-stranded closed circular DNA, and second, the primer sequence must be in close proximity to this template. We used an adenovirus vector *Ad E1::HO Site B*, containing the HO cleavage site inserted in early region 1. To produce a template molecule specific for HO cleavage sites, we produced an 81 nt "open circle" oligonucleotide with ends complementary to 30 nt contiguous sequence within the HO cleavage site in *Ad E1::HO Site B*. Annealing of this oligonucleotide to denatured HO cleavage site will

result in formation of a noncovalently closed circle with the remaining 51 bases of the open circle oligonucleotide forming an unhybridized loop (Fig. 1A). In the next step, DNA ligase is used to covalently join the 5' phosphate and 3' hydroxyl group, which are in close proximity after the open circle anneals to the HO cleavage site, resulting in a covalently-sealed circle. If the circular DNA is annealed to a cleaved HO site, the 3'OH group of the cleaved HO site can serve as a primer for amplification of the ligated circle template sequence by a strand-displacing DNA polymerase. The DNA polymerase progresses around the ligated circle continuously until the 81 bases are amplified hundreds of times, resulting in the in situ amplification product consisting of tandem repeats complementary to the sequence of the

Figure 4. In situ RCA after adenovirus coinfection. 293 cells grown on 8-well culture slides were coinfected with *Ad E1::HO Gene* and *Ad E1::HO Site B* with m.o.i. of approximately 0.04. 18 hours after infection, ligation, RCA and incubation in blocking buffer were performed first, then adenovirus-infected cells were identified by IF using rabbit anti-adenovirus late structural protein antiserum. FITC-conjugated anti-rabbit IgG was added to the decoration buffer containing Cy3-labeled decorator oligonucleotides. Nuclei were stained with DAPI.



Figure 5. Simultaneous in situ RCA and IF of γ -H2AX after adenovirus infection. 293 cells grown on 8-well culture slides were coinfected with Ad E1::HO Gene and/or Ad E1::HO Site B with m.o.i. of 5. 18 hours after infection, γ -H2AX IF was carried out prior to RCA, after the cells were fixed and permeabilized. Briefly, cells were incubated in blocking buffer for 30 min at 37°C. Then, cells were incubated with mouse anti-human γ -H2AX (phospho-Histone H2AX (Ser139)) monoclonal antibody and FITC-conjugated anti-mouse IgG. After slides were washed, cells were incubated in 10mM EGS at 37°C for 45 min. Cells were washed in 2xSSC, then in situ RCA was carried out as described in "Material and Methods". RCA products were labeled by Cy3. Nuclei were stained with DAPI.



Figure 6. In situ RCA after transient transfection. 293T cells were transiently transfected with pcDNA vector, pcDNA-HOsite and/or pcDNA-HOendo, or mock transfected. Cells were fixed approximately 48 hrs after transfection and in situ RCA performed as described in "Materials and Methods". RCA products were labeled by Cy3. Nuclei were stained with DAPI.

ligated circle.⁹ RCA product can be visualized with complementary fluorophore-labeled decorator oligonucleotides (Fig. 1A). In contrast, if the HO site in the *Ad E1::HO Site B* genome has not been cleaved, the open circle can anneal to the denatured HO site and be ligated, but there is no 3' end in *Ad E1::HO Site B* DNA proximal to the ligated circle to serve as a primer for DNA amplification. Hence, no signal would occur (Fig. 1B). Since the amplified products remain at the site where the cleavage and DNA synthesis occur, the RCA signal should localize to the DSB in situ.

Analysis of ligation and RCA in solution. We first tested the method in solution. We determined whether a "target" oligonucleotide encompassing 30 nt of Ad E1::HO Site B including part of the HO cleavage site would hybridize to the open circle oligonucleotide and serve as a scaffold for ligation of the open circle. The reaction products were analyzed by urea-PAGE (Fig. 2A). Slower-migrating ligated circles were observed only when both target and open circle were present. With relatively large amounts of target oligonucleotides as scaffold, intermolecular ligation of the circle molecules, besides the intramolecular ligation, can lead to the production of a ladder of concatemers. A portion of the ligation mixture was next used as a source of template and primer for RCA in the presence of Sequenase DNA polymerase, E. coli SSB, which stimulates Sequenase amplification activity, and dNTPs. Analysis of the reaction mixture by alkaline-agarose gel electrophoresis revealed a heterogeneous mixture of extended DNA molecules, produced only in the presence of ligation mixture. A significant fraction of the molecules were greater than 23 kb in length (Fig. 2B).

In situ RCA. We proceeded to carry out in situ RCA by first infecting HEK293 cells, which express endogenous adenoviral E1 proteins and thus allow the replication of E1-defective adenoviruses, with engineered adenoviruses. The recombinant virus *Ad E1::HO Gene* carries HO endonuclease coding sequences derived from *Saccharomyces cerevisiae* in the E1 region, under transcriptional control of the cytomegalovirus immediate early promoter. This should enable expression of the HO gene shortly after infection. *Ad E1::HO Site B* carries a *Bam*HI to *SmaI* fragment containing the MATa HO site from pWJ421. Coinfection of 293 cells with both viruses results in cleavage of the HO site to steady state levels of some 10 to 30% of the total viral genomes (data not shown), similar to that observed with the original *AdE1::HO site* virus.¹³

Approximately 18 hours after infection of 293 cells under low m.o.i. condition, RCA was performed directly on 8-well culture slides. Antiserum recognizing adenoviral late structural proteins was used to stain cells infected with either or both viruses, since the expression of adenoviral E1A in 293 cells allows late protein expression of E1 deleted viruses.

Punctate RCA signals were seen exclusively in cultures that had been coinfected with adenoviruses encoding HO endonuclease and HO cleavage site (Fig. 3A and D). Such signals were not observed in cells that were mock-infected (Fig. 3M) or infected with either the site or endonuclease virus only (Fig. 3E, I). Moreover, RCA signals were only found in the subset of cells that expressed adenoviral antigen (FITC staining) (Fig. 3D and Fig. 4), and

were never seen in antigen-positive cells in cultures infected with site virus or endonuclease virus only. Negative controls for FITC-IF, in which rabbit IgG was used instead of anti-adenovirus antigens, produced no IF signal (data not shown).

The RCA signals were restricted to the nuclei (Fig. 3D and Fig. 4), which might be associated with the nuclear replication of adenovirus. The choice of fixation methods affected the final results of RCA. No RCA signal was observed in coinfected cells that were fixed with freshly made methanol/acetic acid (3:1 vol:vol) instead of 4% PFA. While denaturation of cellular DNA by heating cells in 70% formamide in 2x SSC at 70°C for 2 min was compatible with strong RCA signals, alkaline denaturation using 0.1 M NaOH for 2 min diminished the RCA signal.

Simultaneous RCA and IF of γ -H2AX in situ. To study cellular signaling pathways activated in response to DNA DSBs, we sought to combine in situ RCA, which localizes the DSBs, and IF staining for known or candidate DNA damage signaling proteins. Formation of foci by γ -H2AX, a form of histone H2AX phosphorylated at Ser139, occurs early in response to γ irradiation. The number of γ -H2AX foci approximates the number of expected DSBs. When DSBs were induced at defined subnuclear regions by pulsed microbeam laser, γ -H2AX foci appear at the damage traces.^{2,18,19} These results indicate that γ -H2AX localizes to DNA DSB sites. Other forms of DNA damage induce only low level of γ -H2AX.²⁰ Initial efforts to combine RCA and IF of γ -H2AX, which were carried out by doing ligation and RCA first, followed by incubation with primary antibody, and then mixing of secondary antibody and RCA decorator, were not successful. Under these conditions, the γ -H2AX (data not shown).

We next tested EGS, a crosslinker that favors intermolecular crosslinking, in an effort to crosslink the antigens and antibodies after γ -H2AX staining. This was followed by in situ RCA. Not only did EGS treatment stabilize the γ -H2AX signal, but the RCA signal was also brighter (Fig. 5A and B). For these experiments, a higher m.o.i of approximately five was used. No RCA signal was observed in cells infected with either *Ad E1::HO Site B* only, or cells that were mock-infected (Fig. 5E and I). It is noteworthy that γ -H2AX staining was induced with adenovirus infection (compare Fig. 5B and F, to mock-infected Fig. 5J), since staining patterns were similar in coinfected cells and cells infected by *Ad E1::HO Site B* only (Fig. 5B and F). This suggested that adenoviral infection itself might up-regulate this H2AX phosphorylation. However, there was no obvious colocalization of RCA signal and γ -H2AX staining.

Since these results suggested that adenovirus infection would mobilize a DNA damage response independent of HO endonuclease cleavage, we investigated an alternative approach, in which cells were transiently transfected with plasmids carrying the HO endonuclease cleavage site, or encoding HO endonuclease. Since the backbone of pcDNA plasmid has an SV40 replication origin, transfection of cells expressing SV40 large T antigen, should result in the amplification of the plasmids, yielding many copies of the HO site. Focal RCA signals were obtained upon transfection of 293T cells with pcDNA-HOsite. However, these signals were produced regardless of the presence of HO endonuclease (Fig. 6). Hence, there is a fundamental difference between the transient plasmid transfection format and adenovirus-mediated transfer of HO sites and HO genes.

DISCUSSION

Improved methods for precisely localizing DNA damage sites in intact cells in situ would facilitate our understanding of DNA damageactivated signaling pathways, especially the damage sensors. Introduction of yeast HO endonuclease and its recognition site into cultured mammalian cells makes it possible to induce sequencespecific DNA DSBs.¹³ The unique identification of RCA signals in cultures doubly infected with viruses carrying endonuclease and cleavage sites, and the nuclear localization of these signals, suggests that the sites of RCA were indeed sites of DSBs induced by this system. We also demonstrated that in situ RCA and immunofluorescence can be performed simultaneously on the same sample, with the preservation of strong signals of both the RCA and the protein fluorescence.

Various methods have been adopted to induce DNA DSBs and study the DNA damage response pathways. In one study, ultrasoft X-rays and microfabricated irradiation masks were used to induce DNA damage in defined subnuclear areas. Subsequently, DSBs were end-labeled with the incorporation of BrdU by terminal TdT.⁷ In other studies, cellular DNA was sensitized to form DSBs at sites of BrdU incorporation upon ultraviolet A (UVA, 390 nm) irradiation when cells were grown in the presence of low concentration of BrdU. Hoechst dye 33258, a DNA staining dye which has substantial absorption at 390nm, was used to achieve the visualization of subnuclear region irradiated.^{2,18,19,21} IF was used to show that DNA damage signaling proteins, such as Mre11⁷ and γ -H2AX^{2,18,19} were localized to the sites of DSBs induced by X-rays or UVA. Inducible budding yeast HO endonuclease or bacterial restriction enzyme, such as EcoRI, has been used to induce DSBs in yeast cells. Southern blot was used to confirm the breaks, ChIP to study the proteins associated with the DNA breaks.^{5,6} Although the aforementioned physical methods to induce DNA DSBs, such as ionizing radiation, X-rays and UVA, make it possible to study the temporal nature of DNA breaks and cellular response, they may produce various kinds of alterations of DNA, including modification of the base and sugar residues and single-strand breaks or DSBs of DNA backbone. ChIP does not have the advantage of detecting proteins associated with DNA breaks, since protein associated with intact chromatin also gives a positive signal. The precise localization of the DNA DSBs achieved by the in situ RCA method will help shed light on the details of DNA damage responses and DNA repair.

Our system, which uses adenoviruses as vectors to infect permissive cells, has several advantages. Adenovirus infection is simply carried out, and does not require access to specialized equipment for irradiation. We have shown that the system is compatible with simultaneous detection of proteins, especially if protein cross-linking is used to stabilize the antibody-antigen complex. Alternatively, green fluorescent protein conjugates have been very useful in localization of DNA damage response proteins,⁶ and should be compatible with this system.

Nonetheless, there are some drawbacks to the system in its present form. The greatest difficult is that infection of cells with adenovirus induced accumulation of γ-H2AX. Similarly, we observed that adenovirus infection induces phosphorylation of Chk2, an intermediary in DNA damage signaling (Hooker G, Stern DF, unpublished observations). Both results suggest that infection with these DNA viruses induces a DNA damage response, as has also been observed for other DNA viruses.^{22,23} Adenovirus has a linear double-stranded DNA genome. It is possible that infected cells sense large amounts of exogenous viral DNA as DSBs, thus activating the response pathway, including the phosphorylation of histone H2AX. Adenoviral oncoproteins are capable of causing the reorganization and proteasomal degradation of Mre11-Rad50-NBS1 complex important for the signal transduction of DNA damage, as well as DNA repair.²⁴ The activation of DNA damage responses by adenovirus does place some limits on the utility of the dual damage and protein detection system, since damage response proteins are likely to accumulate at nonDSB sites induced by adenovirus. The failure of γ -H2AX to colocalize with RCA signals may be symptomatic of another issue, the fundamental differences between adenovirus genome chromatin and endogenous chromatin.²⁵

In order to produce a virus-independent strategy, we assayed HO site cleavage in cells transiently transfected with plasmids that have an SV40 replication origin. An RCA signal was produced when the HO site genome was transfected alone, but production of the RCA signal did not require cotransfection with the endonuclease gene. Transfected DNA undergoes random end-end joining, as well as homologous recombination. Since efficient RCA requires the location of the template site near a DNA end that can serve as primer, we hypothesize that the HO-independent signal was produced as a result of the large number of random free ends that occurs during processing of transfected DNA,²⁶ a small fraction of which would have been randomly broken or cleaved close enough to the HO site so that RCA can proceed. Large amounts of DNA might lead to complicated recombination events with the occurrence of broken ends. Furthermore, SV40 large T antigen can disrupt the focus formation of Mre11 in both T-antigen immortalized cells and in cells transiently transected with T-antigen.²² Therefore, it is also possible that SV40 T-antigen and the abnormal DNA damage response pathway in our 293T system contribute to the appearance of the nonspecific RCA signal. It should be straightforward to bypass these problems in future work by producing cell lines that are stably transfected with the HO cleavage site, and harbor an HO endonuclease gene under the control of an inducible promoter.

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