Localization of hRad9, hHus1, hRad1, and hRad17 and Caffeine-sensitive DNA Replication at the Alternative Lengthening of Telomeres-associated Promyelocytic Leukemia Body*

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Telomere maintenance is essential for continued cell proliferation. Although most cells accomplish this by activating telomerase, a subset of immortalized tumors and cell lines do so in a telomerase-independent manner, a process called alternative lengthening of telomeres (ALT). DNA recombination has been shown to be involved in ALT, but the precise mechanisms remain unknown. A fraction of cells in a given ALT population contain a unique nuclear structure called APB (ALT-associated promyelocytic leukemia (PML) body), which is characterized by the presence of telomeric DNA in the PML body. Here we describe that hRad9, hHus1, and hRad1, which form a DNA clamp complex that is associated with DNA damage, as well as its clamp loader, hRad17, are constitutive components of APB. Phosphorylated histone H2AX (γ-H2AX), a molecular marker of double-strand breaks (DSBs), also colocalizes with some APBs. The results suggest that telomeric DNAs at APBs are recognized as DSBs. PML staining and fluorescence in situ hybridization analyses of mitotic ALT cells revealed that telomeric DNAs present at APBs are of both extrachromosomal and native telomere origins. Furthermore, we demonstrated that DNA synthesis occurs at APBs and is significantly inhibited by caffeine, an inhibitor of phosphatidylinositol 3-kinase. Taken together, we suggest that telomeric DNAs at APBs are recognized and processed as DSBs, leading to telomeric DNA synthesis and thereby contributing to telomere maintenance in ALT cells.

Telomeres are specialized structures at the ends of linear chromosomes of eukaryotic cells. They are composed of tandem arrays of repetitive sequences, telomere repeats, as well as specific binding proteins. The telomeric DNA-protein complex is required for realizing the stable inheritance of chromosomes by preventing the degradation and rearrangement of chromosomal ends. During conventional DNA replication, telomeric DNA is not completely replicated because of the inability of the lagging strand synthesis to start from the very end of DNA (the end replication problem). Because of this, telomeric DNA is shortened every time a cell divides. When the telomeric DNA length becomes critically reduced, the cell enters a state called replicative senescence, in which the cell stops growing irreversibly (1). Tumor cells that undergo a large number of cell divisions need to bypass this limitation of cell proliferation. Most tumor cells realize this by activating telomerase, a specialized reverse transcriptase that synthesizes telomeric repeats (2). However, a small yet significant number of tumor cells bypass the end replication problem in a telomerase-independent manner, the process of which is called alternative lengthening of telomeres (ALT)1 (3).

Several unique features are found in ALT cells compared with telomerase-positive tumor cells (reviewed in Ref. 4). It appears that ALT is more often present in cell lines and tumors of mesenchymal origin, such as osteosarcomas and in vitro transformed human fibroblasts, than in those of epithelial origin. Telomere lengths of ALT cells are characterized by heterogeneity: some telomeres are extremely long; at the same time, others are very short or even undetectable in a single cell. When ALT cells are fused with normal cells or telomerase-positive tumor cells, the ALT mechanism is lost in the resulting cell hybrid, suggesting that one or more recessive gene mutations are involved in ALT (5). The one or more genes responsible for ALT remain unknown, however. In budding yeast, telomerase-defective cells maintain telomeres by DNA recombination (6). Similarly, it was demonstrated that marker DNA cassettes inserted into particular telomeres in ALT cells are duplicated into other telomeres through gene conversion (7). Therefore, DNA recombination is a phylogenetically conserved backup mechanism for maintaining telomeres in telomerase-negative cells. However, the molecular details of ALT are not well understood.

Promyelocytic leukemia (PML) body (alternatively referred to as POD for PML oncogenic domains) is a doughnut-shaped speckled structure in the nucleus (reviewed in Ref. 8). Typically, 10–30 PML bodies are contained in a single cell, but their sizes and numbers change dramatically in various situations. PML protein, which was originally found as a fusion partner of the chimeric protein with the retinoic acid receptor produced in acute promyelocytic leukemia, is a major component of PML.

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¶ The abbreviations used are: ALT, alternative lengthening of telomeres; PML, promyelocytic leukemia; APB, ALT-associated PML body; DSB, double-strand break; IF, immunofluorescence; FISH, fluorescence in situ hybridization; BrdUrd, 5-bromo-2′-deoxyuridine; PNA, peptide-nucleic acid; PCNA, proliferating cell nuclear antigen; RFC, replication factor C; HA, hemagglutinin; PBS, phosphate-buffered saline; RSZ, replication slow zone.

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bodies. It has been reported that a large number of proteins, including p53, pRB, DAXX, and CBP, are contained in PML bodies. PML bodies have been implicated in diverse biological functions: they may regulate transcription, apoptosis, cellular proliferation, senescence, and tumor suppressor functions. Interestingly, proteins involved in DNA metabolism, such as BLM, Mre11, and NBS1, are also contained in PML bodies (9–11). BLM, a RecQ-type helicase encoded by the gene mutated in the Bloom syndrome, which is characterized by high frequencies of sister chromatid exchanges and cancer susceptibility. NBS1 is encoded by the gene mutated in the Nijmegen breakage syndrome that is associated with genetic instability and cancer predisposition. NBS1, Mre11, and Rad50 form a complex called the MRN complex, which plays a critical role in DNA damage sensing, repair, and checkpoint. These results suggest that PML bodies are also involved in genomic stability.

A fraction of cells in a given ALT population possesses enlarged PML bodies called APBs (ALT-associated PML bodies) (12). APBs are unique because of the presence of a large amount of telomeric repeats as revealed by fluorescence in situ hybridization (FISH) or the immunological detection of telomeric DNA-binding proteins TRF1 and TRF2. APBs contain PML, Mre11, NBS1, Rad50, and BLM that normally exist in PML bodies. In addition, APBs contain the telomere-associated protein Rap1 and the recombination proteins Rad51, Rad52, and BRCA1, which are not normally present in PML bodies (12–15). Furthermore, 5-bromo-2′-deoxyuridine (BrdU) incorporation was detected at the NBS1 foci in a small fraction of growing ALT cells, suggesting that DNA is synthesized at APBs (15). However, it is not known whether telomeric DNA is synthesized and maintained by the DNA recombination reaction at APBs or not.

Damaged sites of DNA are recognized by a mechanism called the damage checkpoint, leading to general cellular responses, including cell cycle arrest and DNA repair (reviewed in Ref. 16). Proteins required for the damage checkpoint were identified through genetic studies in yeast at first, and most of them were conserved in eukaryotes, including humans. A class of damage checkpoint proteins function in sensing damaged DNA, including mammalian ATM and ATR protein kinases, and human homologs of the fission yeast Rad9-Hus1-Rad1 complex and Rad17 protein (hRad9, hHus1, hRad1, and hRad17) (17). ATM and ATR play a major role in transducing DNA damage checkpoint signals. Rad9, Rad1, and Hus1 proteins form a trimeric ring-shaped complex that is closely related to the PCNA complex, a DNA sliding clamp of the DNA replication machinery (18–23). PCNA is loaded onto DNA by a clamp loader, replication factor C (RFC) (24). RFC is composed of four small subunits (RFC2–5) and one large subunit (RFC1 p140). Rad17 shares sequence homology with RFC1 and physically interacts with the four small subunits of RFC, suggesting that Rad17 forms an alternative type of RFC-like complex by substituting RFC1 (Rad17-RFC) (25–27). Indeed, the Rad9-Hus1-Rad1 complex is loaded onto damaged DNA in a Rad17-dependent manner (28). Interestingly, strains mutated in the rad1, hus1, rad9, or rad17 genes in fission yeast and mrt-2 (encoding Rad1 homolog) in nematode show telomere shortening, suggesting that these checkpoint proteins are also involved in telomere maintenance (29–31). Here we demonstrate that these proteins are colocalized specifically at telomeric DNAs and PML bodies in ALT cells. We also present evidence that the DNA damage checkpoint is operating in the APB-positive ALT cells.

EXPERIMENTAL PROCEDURES

Plasmids—cDNA fragments encoding hRad9 and hRad1 were a gift from Dr. Howard Lieberman. hHus1 cDNA was obtained as the expressed sequence tag clone AW518029 from Inegeo. Genomics, Inc. pcDNA3-hRad9-HA, pcDNA3-hRad1-HA, and pcDNA3-hHus1-HA were constructed from pcDNA3 (Invitrogen) to produce HA-tagged hRad9, hRad1, and hHus1 proteins, respectively.

Cell Culture—GM639, GM847, BPT-3B, and W138VA132RA were a gift from Dr. Roger Reddel. All cells used in this study were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Transformation was performed using LipofectAMINE (Invitrogen), and colonies were isolated after incubation for 2 weeks in a medium containing 800 μg/ml G418 (Nacalai). Cells for microscopic analyses were grown on coverslips. To obtain mitotic cells for chromosome spreads, cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum containing 0.1 μg/ml Colcemid (Roche Applied Science) for 8–10 h and harvested by media flow.

Antibodies—Primary antibodies and dilution ratios for immunofluorescence (IF) analysis were as follows: anti-mouse Rad9 antibody (1:200), anti-TRF2 rabbit antibody (1:200), and anti-PML mouse monoclonal antibody (1:500) from Santa Cruz Biotechnology; anti-NBS1 rabbit antibody (1:290) from Oncogene; anti-human Rad17 rabbit antibody (1:200) from Cell Signaling; anti-γ-H2AX monoclonal mouse antibody (1:500) from Upstate; anti-BrdUrd monoclonal mouse antibody (1:500) from Roche Applied Science; and anti-HA monoclonal mouse antibody clone 12B12 (1:500) from Covance/BAbCO. Anti-TRF1 rabbit antibody was raised in our laboratory. Secondary antibody against rabbit or mouse IgG conjugated with Alexa488 was purchased from Molecular Probes. Cy3-labeled anti-rabbit IgG antibody and anti-mouse IgG antibody were obtained from Amersham Bioscience. Cy5-conjugated secondary antibodies were obtained from Jackson ImmunoResearch. All the secondary antibodies were used after 1:1000 dilution.

Immunofluorescence and FISH Experiments—IF staining and telomere fluorescence were performed essentially as described (32–35) with some modifications. Mitotic spreads of chromosomes were prepared by Cytospin as described (36), except cells were swollen in 75 mm KCl. Cells were prepermeabized prior to fixation with 0.1% Triton X-100 in T buffer (20 mM HEPES-KOH (pH 7.4), 50 mM NaCl, 3 mM MgCl2, and 0.3 mM sucrose). After two rounds of PBS washes, cells were fixed with 3% paraformaldehyde in PBS for 10 min on ice. The samples were washed with PBS and residual fixatives were quenched in PBS containing 0.1 μg/ml glycine for 15 min. Permeabilization was conducted for 5 min at room temperature with 0.5% Triton X-100 in T buffer, followed by two rounds of PBS washes. Samples were treated with the blocking buffer (0.1% skim milk, 0.1% bovine serum albumin in PBS) for 15 min before incubating with antibodies. When anti-BrdUrd antibody was used, cells were incubated with 50 units/ml DNase I (Takara) for 20 min at room temperature in the blocking buffer. One-hundred microliters of the blocking buffer containing primary antibodies was overlayed onto a coverslip and incubated for 2 h at room temperature. After washing three times with PBS, cells were treated with secondary antibodies in the blocking buffer for 1 h at room temperature. Then, after washing three times with PBS, samples for IF experiments were counterstained for DNA with 1 μg/ml Hoechst 33342 (Nacalai) in PBS. Samples intended for simultaneous IF and FISH experiments were subjected to further processing before DNA staining.

Postfixation prior to FISH staining was performed at room temperature for 15 min in 3.7% formalin in PBS, followed by washing with PBS. The fixation reaction was quenched with 0.1 μg/ml glycine in PBS, followed by washing with PBS. Coverslips were overlaid with 100 μl of hybridization buffer (50% formamide, 10% dextran sulfate, 5X Denhardt’s solution in 2X SSC) containing 0.3 μg/ml Cy3-conjugated 5′-CCCTAA-3′ PNA (Sawara) and 3′ PNA. The coverslips were incubated at 80°C for 3 min to denature DNA and then cooled to and incubated at room temperature for more than 6 h to hybridize the probe. Then, the coverslips were washed with 50% formamide in 2X SSC for 30 min followed by two rounds of washing with 2X SSC for 5 min. DNA was counterstained with 1 μg/ml Hoechst 33342 in PBS. All samples were mounted in Vectorshield (Vector) with 4′-6-diamidino-2-phenylindole (DAPI) and a DeltaVision restoration microscope (Applied Precision), with a charge-coupled device camera (Photometrics). The images were processed by Graphic Converter (Lemke Software) and Photoshop (Adobe) software.

RESULTS

Colocalization of hRad9 and Telomeric DNA at PML Bodies Specifically in ALT Cell Lines—To investigate the function of hRad9 in ALT cells, we first examined whether hRad9 localizes at telomeres or not. We detected simultaneously hRad9 and
PML proteins, and telomeric DNA by indirect IF and FISH staining, respectively. We tested five ALT cell lines (U2-OS, GM847, SUSM-1, SaOS-2, and WI38 VA13/2RA) and three types of telomerase-positive cell lines (GM639, HOS, and BFT-3B). hRad9 (green) and PML (red) were stained with specific primary antibodies and Alexa488- or Cy5-labeled secondary antibody. FISH staining of telomeric DNA (red) was simultaneously performed with Cy3-labeled telomeric PNA as probe. Counterstaining of nuclear DNA was performed with Hoechst 33342 (blue). Cells were treated with 0.1% Triton X-100 containing buffer prior to fixation to strengthen signals of insoluble and/or chromatin-associated proteins. Superimposed images of hRad9 (green), telomeric DNA (red), and DNA (blue) and hRad9 (green), PML (red), and DNA (blue) are shown as indicated, where colocalized signals of hRad9 with telomeric DNA or PML appear as yellow foci. Bar, 10 μm.

Fig. 1. Colocalization of hRad9, telomeric DNA and PML at nuclear bodies specifically in ALT cell lines. Indirect IF combined with FISH studies were performed in five types of ALT cell lines (U2-OS, GM847, SUSM-1, SaOS-2, and WI38 VA13/2RA) and three types of telomerase-positive cell lines (GM639, HOS, and BFT-3B). hRad9 (green) and PML (red) were stained with specific primary antibodies and Alexa488- or Cy5-labeled secondary antibody. FISH staining of telomeric DNA (red) was simultaneously performed with Cy3-labeled telomeric PNA as probe. Counterstaining of nuclear DNA was performed with Hoechst 33342 (blue). Cells were treated with 0.1% Triton X-100 containing buffer prior to fixation to strengthen signals of insoluble and/or chromatin-associated proteins. Superimposed images of hRad9 (green), telomeric DNA (red), and DNA (blue) and hRad9 (green), PML (red), and DNA (blue) are shown as indicated, where colocalized signals of hRad9 with telomeric DNA or PML appear as yellow foci. Bar, 10 μm.

We therefore concluded that hRad9 is a constitutive component of APBs in ALT cells. Signals of telomeric DNA colocalizing with hRad9 were likely to be more intense than hRad9-negative ones. In contrast to ALT cells, hRad9 was not associated with telomeric DNA in telomerase-positive cells (Table II). Unexpectedly, however, a small yet significant fraction of hRad9 foci were overlapped with PML signals in telomerase-positive cells, although the extent was less than that observed in ALT cells (from 18.1% in HOS to 37.3% in GM639 cells, Table II). The implication of this observation is not known at the moment.

**Localization of hRad9-associating Proteins at APB—Rad9 associates with Rad1 and Hus1 to form a PCNA-like DNA-
sliding clamp. Upon DNA damage, this clamp complex is loaded onto chromatin by an RFC-like complex containing hRad17. To test whether the other sliding clamp molecules and the clamp loader molecules are also present at APBs, we examined the localization of hHus1, hRad1, and hRad17 in ALT cells. U2-OS cell lines that expressed HA-tagged hHus1, hRad1, or hRad9 individually were constructed. Following 0.1% Triton X-100 treatment, the localization of the HA-tagged proteins with respect to that of TRF1 was analyzed by simultaneous indirect IF staining using anti-HA and anti-TRF1 antibodies (Fig. 2A). We observed that hHus1-HA, hRad1-HA, and hRad9-HA were all colocalized with the large TRF1-positive foci that most likely represented APBs. The percentages of cells possessing TRF1 foci that colocalized with hHus1-HA, hRad1-HA, or hRad9-HA were comparable to each other (10.8% for hHus1-HA, 8.0% for hRad1-HA, and 7.2% for hRad9-HA; n = 250).

We further examined the localization of endogenous hRad17. ALT cells, and telomerase-positive cells were prepermeabilized by 0.1% Triton X-100 treatment and stained for hRad17 and PML and telomeric DNA with indirect IF and FISH techniques, respectively (Fig. 2B). Similar to hRad9, hRad17 showed detergent-resistant nuclear foci in a fraction of populations of both ALT cells and telomerase-positive cells (from 6.1% in HOS to 37.6% in SUSM-1 cells, Table III). Interestingly, the abundance of hRad17-positive cells was strongly correlated with that of hRad9-positive cells (compare Tables I and III). Most hRad17-positive ALT cells were APB-positive (Table III), and most hRad17 foci in ALT cells were overlapped with APBs (Table IV). In contrast, hRad17 foci in telomerase-positive cells were partly overlapped with PML signals, but were not with telomeric DNA signals (Table IV). These quantitative analyses underscore the intimate behaviors of detergent-resistant hRad9 and hRad17. However, these behaviors are different between ALT and telomerase-positive cells (whether or not present at telomeric DNA). Taken together, these results indicate that the clamp proteins hRad1, hHus1, and hRad9 as well as the clamp loader protein hRad17 are constitutive components of APBs.

Localization of γ-H2AX at APB—It has been demonstrated that APBs contain a variety of proteins, including Rad51, Rad52, RPA (replication protein A) (12), NBS1, Mre11 (15), Rap1, and BRCAl1 (14). We also confirmed that NBS1 is colocalized at APBs that contain telomeric DNA and PML (Fig. 3A). Together with Rad50, NBS1 and Mre11 form a complex (the MRN complex) that participates in the recognition and processing of DSBs. Upon DNA damage, locations of the MRN complex in the nucleus are reorganized into focal structures called irradiation-induced foci (10, 37), hRad9, hHus1, and hRad1 also associate with chromatin upon DNA damage (28, 38). It is therefore possible that telomeric DNAs at APBs in ALT cells are recognized as damaged sites by cells. Phosphorylated histone H2AX (γ-H2AX) is one of the molecular signals that accumulate at damaged sites in the earliest time course of DSB repair (39, 40). It is well established that γ-H2AX nuclear foci are formed upon DNA damage. However, we found that detergent-resistant γ-H2AX nuclear foci are present in a fraction of the interphase nuclei of both ALT and telomerase-positive cells that were not subjected to any DNA damage-inducing conditions (Fig. 3B). The size of these γ-H2AX nuclear foci was smaller than those typically observed when DSBs were induced. The percentage of interphase cells possessing at least one γ-H2AX nuclear focus ranged from as low as 7.3% in GM639 cells (telomerase-positive) to as high as 38% in GM847 and SUSM-1 cells (both ALT) (Table V). A similar observation was reported previously (39). Interestingly, we found that fractions of γ-H2AX nuclear foci in ALT cells were colocalized with TRF1 (8.9% of total γ-H2AX nuclear foci were overlapped with TRF1 in U2-OS, n = 123 foci; 14.3% in GM847, n = 119; and 10.7% in SUSM-1, n = 121), whereas the γ-H2AX-positive foci in telomerase-positive cells were not (0% in GM639, n = 122; and 0% in HOS, n = 138). When hRad9 or NBS1, and telomeric DNA were simultaneously detected by indirect IF and FISH techniques, respectively, some of the γ-H2AX-positive foci in ALT cells were found to be overlapped with APBs (defined by the presence of telomeric DNA and hRad9 or NBS1) (Fig. 3, C and D). It was noted that not all APBs (telomeric DNA- and NBS1-double-positive foci) were γ-H2AX-positive (22.4% of APBs contained γ-H2AX in U2-OS, n = 113; 47.0% in GM847, n = 121; and 66.4% in SUSM-1, n = 118). Moreover, less than half of the γ-H2AX-positive ALT cells contained γ-H2AX- and TRF1-double-positive foci (Table V). Therefore, γ-H2AX foci are partially overlapped with APBs in ALT cells. This is in contrast to hRad9 or hRad17 foci, most of which are associated with APBs (Tables II and IV). Taken together, the results suggest that the APBs contain DNA structures recognized by DNA damage checkpoint proteins, and a fraction of them induce the localization of γ-H2AX.

Presence of Native Telomeres and Extrachromosomal Telomeric DNAs at APB—It is known that extrachromosomal telomeric DNA is present in ALT cells (41–43). Therefore, it is important to know if telomeric DNAs at APBs detected by FISH and TRF1-staining are derived from native telomeres or extrachromosomal telomeric DNAs. To this end, we prepared metaphase chromosomes from ALT cells and stained the chromosomes with telomeric DNA and anti-PML antibody simultaneously. It was reported previously that APBs remain in mitotic ALT cells (44). We observed that most PML signals in mitotic ALT cells appeared as extrachromosomal elements that were positive for telomeric DNA FISH staining (Fig. 4). However, we also found that a significant number of PML signals were localized at telomeric DNA-positive native telomeres. Although it is not certain that the PML-positive structures in mitotic ALT cells are directly derived from APBs in the interphase cells, the results suggest that APBs contain both native telomeres and extrachromosomal telomeric DNAs.

Localization of BrdUrd Foci at APB—DSBs are formed by a variety of treatments such as radiation and chemicals. In addition, it is known that the replication fork stall entails DSBs under certain conditions. The damage checkpoint proteins recognize DSBs and transduce signals for activating repair pathways. DNA synthesis is an important step in the repair mechanism, for example, in homologous recombination and in restarting the stalled replication fork. Together, it is possible

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**TABLE II**

**CheckPoint Rad Proteins at APB in ALT Cells**

| Cell line | Telemic DNA (+) | PML (+) | APB(+) | No. of foc |
|-----------|-----------------|---------|--------|-----------|
| ALT cell line | | | | |
| U2-OS | 91.0 | 94.0 | 91.0 | 100 |
| GM847 | 96.3 | 100 | 96.3 | 80 |
| SUSM-1 | 94.0 | 94.8 | 94.0 | 116 |
| Saos-2 | 91.6 | 92.6 | 90.5 | 95 |
| W138 VA13/2RA | 86.6 | 83.2 | 83.2 | 119 |

* APB is defined as a colocalized focus of telomeric DNA and PML.
that DNA synthesis is involved in telomere maintenance at APBs. To test this possibility, we detected the site of de novo DNA synthesis in ALT cells by pulse labeling with BrdUrd.

Asynchronized ALT cells, U2-OS, GM847 and SUSM-1, were incubated transiently in medium containing BrdUrd to pulse-label the newly synthesized DNA immediately before fixation. BrdUrd incorporated into the nucleus was detected by indirect IF staining using anti-BrdUrd antibody. APBs were identified based on the criterion that focal structures are positive for both hRad9 IF signals and telomeric DNA FISH signals. Two types of cells showing distinct BrdUrd-staining patterns were observed. In one type, numerous (>50) BrdUrd-positive foci were observed, evidently implicating that the cells were in S phase. Occasionally, BrdUrd-positive foci were overlapped with telomeric DNA in such S phase cells, suggesting that the replication focus replicating telomeric DNA was detected in such cases (Fig. 5, A and B). Interestingly, simultaneous IF staining with anti-hRad9 antibody revealed that hRad9 is present specifically at such telomeric BrdUrd-positive foci, but not at many other nontelomeric BrdUrd-positive foci. Another type of BrdUrd-positive cells contained relatively small numbers (~10) of BrdUrd-positive foci, suggesting that they were not in S phase. Intriguingly, these BrdUrd-positive foci in extra-S phase cells were frequently overlapped with hRad9 and telomeric DNA foci (Fig. 5, C and D), indicating that DNA synthesis takes place in APBs in extra-S phase ALT cells. More than half of the APBs (defined by hRad9 and telomeric DNA-double positive foci) in extra-S phase ALT cells were colocalized with BrdUrd foci (14/22 in 3 cells of U2-OS, 57/82 in 11 cells of GM847, and 36/61 in 7 cells in SUSM-1). Taken together, telomeric DNA synthesis occurs at APBs in both S phase and extra-S phase. The percentages of cells with at least one BrdUrd focus at APBs

| Table III |
| Percentages of cells with hRad17 foci and APBs |
| Percentages of interphase cells containing at least one hRad17 focus (hRad17(+)1), one APB (APB(+)), or both hRad17- and APB-double-positive focus (hRad17(+)1APB(+)) were calculated after observing indicated numbers of cells. |
| Cell line | hRad17(+) | APB(+) | hRad17(+)1APB(+) | No. of cells |
| ALT cell line | | | | |
| U2-OS | 17.0 | 20.8 | 17.0 | 159 |
| GM847 | 28.8 | 32.3 | 28.8 | 198 |
| SUSM-1 | 37.6 | 40.0 | 37.6 | 125 |
| SaOS-2 | 35.3 | 38.7 | 35.3 | 120 |
| W138 VA13/2RA | 32.3 | 35.5 | 30.6 | 124 |
| Telomerase-positive cell line | | | | |
| GM639 | 22.5 | 0.7 | 0 | 138 |
| HOS | 6.1 | 0 | 0 | 132 |
| BFT-3B | 18.1 | 0.7 | 0.7 | 138 |

*APB is defined as a colocalized focus of telomeric DNA and PML.

| Table IV |
| Percentages of foci containing telomeric DNA signal (telomeric DNA(+))1, PML signal (PML(+)), or both telomeric DNA and PML signals (APB(+)) among total hRad17 foci were calculated after observing indicated numbers of hRad17 foci. |
| Cell lines | Telomeric DNA(+) | PML(+) | APB(+) | No. of foci |
| ALT cell line | | | | |
| U2-OS | 97.7 | 96.6 | 96.6 | 87 |
| GM847 | 97.9 | 97.9 | 97.4 | 192 |
| SUSM-1 | 98.8 | 98.8 | 98.8 | 173 |
| SaOS-2 | 90.1 | 93.4 | 90.1 | 91 |
| W138 VA13/2RA | 89.2 | 90.3 | 88.7 | 195 |
| Telomerase-positive cell line | | | | |
| GM639 | 0 | 14.1 | 0 | 64 |
| HOS | 0 | 18.2 | 0 | 11 |
| BFT-3B | 3.5 | 22.8 | 3.5 | 57 |

*APB is defined as a colocalized focus of telomeric DNA and PML.
Fig. 3. Colocalization of γ-H2AX with telomeric DNA and hRad9 or NBS1 at nuclear bodies in ALT cells. A, colocalization of NBS1 with telomeric DNA and PML in GM847 (ALT) cells. Intracellular locations of NBS1 (green), PML (red), and telomeric DNA (blue) were observed after IF and FISH staining using their specific primary antibodies, and Alexa488- or Cy5-labeled secondary antibody and Cy3-labeled telomeric PNA probe. Nuclear DNA was stained with Hoechst 33342 (blue). Images are superimposed as indicated, where colocalized signals of NBS1 with telomeric DNA or PML appear as yellow foci. B, localization of γ-H2AX and TRF1 in GM847 (ALT) and GM639 (telomerase-positive) cells. γ-H2AX (green) and TRF1 (red) were observed after IF staining using their specific primary antibodies and Alexa488- and Cy5-labeled secondary antibodies. Colocalized signals appear yellow. C and D, localization of γ-H2AX with telomeric DNA and hRad9 or NBS1 in GM847 (ALT) cells. A fraction of γ-H2AX foci (green) are colocalized with telomeric DNA (red) and hRad9 (red in C) or NBS1 (red in D). Superimposed signals are shown as indicated, where colocalized signals appear as yellow foci. Bar, 10 μm.

### Table V

| Cell lines          | γ-H2AX (+) | γ-H2AX/γH2AX(+)/TRF1(+) | No. of cells |
|---------------------|------------|--------------------------|-------------|
| ALT cell line       |            |                          |             |
| U2-OS               | 28.7       | 3.3                      | 150         |
| GM847               | 33.7       | 15.3                     | 150         |
| SUSM-1              | 33.0       | 12.7                     | 150         |
| Telomerase-positive cell line | 7.3 | 0.7                     | 150         |
| GM639               | 23.1       | 0.7                      | 150         |

Percentages of interphase cells containing at least one γ-H2AX focus (γ-H2AX(+)) or one γ-H2AX- and TRF1-double-positive focus (γ-H2AX(+/γH2AX(+)/TRF1(+) were calculated after observing indicated numbers of cells.

with respect to total APB-positive cells varied among the ALT cell lines (3.5% in U2-OS to 24.6% in GM847) (Fig. 6).

### Discussion

In this study, we demonstrated that hRad9, hHus1, hRad1, and hRad17 are localized at APBs in ALT cells. These proteins are resistant to detergent treatment, suggesting that they are bound to chromatin. It has been reported that the detergent-resistant chromatin-bound hRad9-hHus1-hRad1 complex is formed only when DNA is damaged, whereas hRad17 constitutively associates with chromatin (28, 38). We also showed that γ-H2AX, a phosphorylated histone H2AX that is specifically formed at the neighbor of DSBs, is localized at a fraction of APBs. It is known that BRCA1, another protein that accumulates at DSBs (39), is colocalized at APBs. Taken together, these data suggest that proficient ATM and ATR activity in ALT cells is not required for APB formation but is required for telomeric DNA synthesis at APBs.

Addendum

A. Nabetani, O. Yokoyama, and F. Ishikawa, unpublished data.
Two possibilities can be envisioned as regards the nature of the telomeric DNAs that are recognized as damaged DNAs at APBs. Telomeres contain the native ends of chromosomal DNAs that escape from being recognized as DSBs. It is possible that telomeric DNAs at APBs assume unusual structures that fail to protect telomeres from activation of the checkpoint. When the telomeric DNA-binding protein TRF2 is inactivated by either a dominant-negative allele or small interference RNA in telomerase-positive cells, the DNA damage checkpoint is activated and telomeric DNA undergoes end-to-end fusion (48, 49). In addition, nuclear foci (telomere dysfunction-induced foci) containing 53BP1, Mre11, γ-H2AX, and phosphorylated forms of ATM and hRad17 are formed at the telomeres (50). It is possible that the protection of telomeric DNA is somehow impaired in ALT cells and the denuded telomeric DNA ends are recognized as damaged. At the same time, the denuded telomeric DNA may serve as a DNA end for inducing the gene conversion between different telomeric DNAs, thereby elongating telomeres in ALT cells. It is known that TRF2 is present at APBs in ALT cells (12). Therefore, the presence or absence of TRF2 per se appears not to be responsible for the telomere dysfunction in this scenario.

Another possibility is suggested from the observation that

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3 A. Nabetani, O. Yokoyama, and F. Ishikawa, unpublished results in the five ALT cell lines.
APBs appear to be closely related to DNA replication. Previously, it was reported that APBs are more frequently observed in G2/M-phase cells than in cells in other cell cycle phases (44). BrdUrd uptake was also observed at NBS1 or TRF1 foci in G2 phase GM847 (15). We further extended the previous observation by showing that BrdUrd-positive APBs (defined as hRad9 and telomeric DNA colocalization) are also present in S-phase cells and that they occur among the numerous replication foci. We could not find any discernible features between the BrdUrd foci at APBs and the BrdUrd-positive replication foci in S-phase, or between BrdUrd-positive APBs in S phase and those in extra-S phase (Fig. 5). Rather, it appears that these BrdUrd-positive foci represent the same structures that remain continuously from S phase to G2 phase. We have shown that telomeric repeats are intrinsically poor substrates for DNA replication in the SV40-based in vitro replication system. When recombinant TRF1 was included in the reaction, most replication forks were stalled within the telomeric repeats (35). It is suggested that the telomere replication, which is intrinsically slow, is stalled more frequently in ALT cells and does not finish when replication at other loci is completed. In this case, the stalled replication fork may be recognized by the checkpoint mechanisms in both S and extra-S phases, and DNA synthesis occurs as a consequence of restarting the conventional DNA replication or inducing the recombination pathway.

In budding yeast, genomic regions called replication slow

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**Fig. 5.** Incorporation of BrdUrd into nuclear bodies positive for hRad9 and telomeric DNA in ALT cells. After cells were labeled with 100 μM BrdUrd for 30 min, they were subjected to IF and FISH analyses. BrdUrd (green) and hRad9 (green) were stained with specific antibodies and Cy5- and Alexa488-conjugated secondary antibodies, respectively. FISH analysis of telomeric DNA (red) was performed with Cy3-labeled telomeric PNA probe. Nuclear DNA was counterstained with Hoechst 33342 (blue). U2-OS (A and C) and GM847 (B and D) cells with more than 50 (A and B) or ~10 (C and D) BrdUrd foci are shown. Merged images of BrdUrd and telomeric DNA, or hRad9 and telomeric DNA are also shown as indicated, where overlapping signals of BrdUrd or hRad9 with telomeric DNA appear as yellow foci. Positions of BrdUrd foci at APBs in A and B are indicated by arrowheads. Bar, 10 μm.

**Fig. 6.** Inhibitory effects of caffeine on BrdUrd incorporation at APBs. Three ALT cell lines in asynchronized cultures were treated with (+caffeine, green bars) or without (−control, blue bars) 5 mM caffeine for 24 h prior to fixation. The numbers of cells analyzed are given in parentheses. Percentages of cells possessing BrdUrd-hRad9-telomeric DNA colocalized foci among the total number of cells with hRad9-telomeric DNA colocalized foci are shown.

### Table VI

| Cell line | hRad9-telomere | BrdUrd | No. of cells |
|-----------|----------------|--------|--------------|
| U2-OS     | 10.8           | 52.9   | 223          |
| U2-OS caf. | 9.5            | 44.3   | 210          |
| GM847     | 24.2           | 48.4   | 161          |
| GM847 caf. | 25.1           | 36.9   | 179          |
| SUSM-1    | 25.0           | 38.7   | 156          |
| SUSM-1 caf. | 24.8          | 39.8   | 181          |

* caf., treatment with 5 mM caffeine.
zones (RSzs) show intrinsic properties of slow replication fork progression in the absence of any extrinsic stress (51). When MECl (homolog of ATR in budding yeast) was disrupted, the fork movement was particularly slow at RSzs, occasionally leading to DSBs. It is possible that telomeric repeats represent one of the RSzs in mammalian cells and are susceptible to DSB formation. The replication stalls and/or thus-formed DSBs at telomeric repeats may be recognized by various proteins sensing these damaged structures and transducing DNA damage checkpoint signals at APBs. In this scenario, the damaged structures recognized by the checkpoint are not the telomeric ends but the replication intermediates that are stalled by replication block at telomeric repeats. It is hypothesized that ALT cells have a DNA replication defect general to genome-wide RSzs or specific to telomeric regions. This hypothesis explains why APB-positive cells appear most frequently in G2 phase cells in a seemingly stochastic manner.

The transient treatment with caffeine affects DNA synthesis at APBs but has no significant effects on global nuclear DNA synthesis or localization of at least two components (hRad9 (Table VI) and NBS1<sup>+</sup>) at APBs. Consistently, it was previously reported that the chromatid association of neither hRad9 nor NBS1 depends on ATM or ATR (52, 53). Because the overall progression of S phase was normal in ALT cells with or without caffeine treatment, it was unlikely that the inactivation of ATM and ATR by caffeine treatment led to reduced DNA synthesis at APB through activating the genome-wide checkpoint mechanism. In budding yeast, Mec1 is required for stabilizing DNA polymerases at the stalled replication fork (54). It is possible that caffeine inactivates ATM and/or ATR in ALT cells, thereby leading to the premature collapse of the stalled replication fork at telomeric repeats. This may result in the failure of resumption of replication and/or homologous recombination-mediated resolution of the stalled replication fork in APBs. Taken together, it is suggested that ATM/ATR-dependent DNA synthesis is important for telomere maintenance in ALT cells.

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Localization of hRad9, hHus1, hRad1, and hRad17 and Caffeine-sensitive DNA Replication at the Alternative Lengthening of Telomeres-associated Promyelocytic Leukemia Body

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