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ALT-associated PML bodies are present in viable cells and are enriched in cells in the G_2/M phase of the cell cycle

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SUMMARY

Telomere maintenance is essential for the unlimited proliferative potential of human cells, and hence immortalization. However, a number of tumors, tumor-derived cell lines and in vitro immortalized cell lines have been described that do not express detectable telomerase activity. These lines utilize a mechanism, termed Alternative Lengthening of Telomeres (ALT), to provide telomere maintenance. A subset of the cells in each ALT cell line contain a novel form of the promyelocytic leukemia nuclear body (PML NB) in which telomeric DNA and the telomere binding proteins TRF1 and TRF2 co-localize with the PML protein, termed ALT-associated PML bodies (AA-PBs). In contrast, in non-ALT, telomerase-positive cell lines these telomeric proteins and the PML NB occupy distinct and separate subnuclear domains. PML NBs have been implicated in terminal differentiation, growth suppression and apoptosis. The role, if any, of AA-PBs in telomere maintenance or culture viability in telomerase negative cell lines is unclear, but it has been suggested that cells containing these structures are no longer viable and are marked for eventual death. We utilized a series of human ovarian surface epithelium (HOSE) cell lines that use ALT for telomere maintenance to determine if AA-PBs are indeed markers of cells in these cultures that are no longer cycling. We show that AA-PB positive cells incorporate BrdU and thus are able to carry out DNA replication. In addition, AA-PBs are present in mitotic cells and the frequency of cells containing these structures is increased when cultures are enriched for cells in the G_2/M phase of the cell cycle suggesting that the formation of AA-PBs is coordinately regulated with the cell cycle. Finally, we demonstrate that the majority of the AA-PB positive cells in the culture are not destined for immediate apoptosis. Taken together the data argue against AA-PBs marking cells destined for death and, instead, raise the possibility that these structures may be actively involved in telomere maintenance via the ALT pathway.

Key words: Alternative lengthening of telomeres, Telomerase, Promyelocytic leukemia nuclear body, Cell cycle regulation

INTRODUCTION

Telomeres are specialized nucleoprotein structures at the ends of linear chromosomes that are required for the stability and complete replication of the termini. The number of divisions a cell may undergo, and hence cellular immortality, is tightly linked to the length of telomeric DNA array. Forced expression of telomerase, the enzyme responsible for maintaining telomeric DNA, prevents both telomere erosion and senescence in several telomerase-negative cell types (Bodnar et al., 1998; Counter et al., 1998; Vaziri and Benchimol, 1998). Likewise, inhibition of telomerase in a number of immortal cell lines reimposes a finite proliferative capacity and, in some cases, resulted ultimately in apoptotic death (Hahn et al., 1999; Herbert et al., 1999; Zhang et al., 1999). In most human tumors telomerase is active (Shay and Bacchetti, 1997) resulting in stabilization of telomeric arrays and presumably unlimited cell division potential.

Although telomerase activity appears the most prevalent mechanism used by tumors to circumvent the telomere length dependent barrier to proliferation, a number of tumors, tumor derived cell lines and in vitro immortalized cell lines have been described that do not contain telomerase activity (Bryan et al., 1997; Bryan et al., 1995; Reddel et al., 1997). These lines utilize a mechanism termed Alternative Lengthening of Telomeres (ALT) to maintain telomeric DNA and circumvent the telomere length dependent limit on proliferation. Telomere length in these cell lines is highly heterogeneous, with repeats ranging in size from >20 kb to <5 kb. The occurrence of ALT is not correlated with the method of immortalization nor with mutations in any known oncogenes or tumor suppressor genes (Whitaker et al., 1995). The molecular mechanism for telomere maintenance in the absence of telomerase remains speculative but based on evidence from telomerase-null strains of the yeast *S. cerevisiae* it has been suggested that recombination between telomeric repeats is the underlying basis for telomere stabilization (Le et al., 1999; Lundblad and Blackburn, 1993).

In ALT positive cell lines, a proportion of cells exhibit co-localization of telomeric DNA and telomere binding proteins with the PML protein in a novel form of the promyelocytic

leukemia nuclear body (PML NB), also called ND10, Kr body and PML oncogenic domains (PODs) (Yeager et al., 1999). These structures have been termed ALT-associated PML bodies (AA-PBs) and are associated with telomere stabilization in ALT positive cell lines in that they first appear in cell lines at the time these lines exhibit telomere lengths consistent with the ALT pathway (Yeager et al., 1999). The association of telomeric components with PML NBs is only observed in cell lines that maintain telomeric DNA in the absence of detectable telomerase activity. In addition, factors involved in replication and recombination such as replication factor A (RPA), Rad51 and Rad52 co-localize with AA-PBs. These observations are consistent with a recombination-based mechanism for telomere maintenance in telomerase negative cell lines.

PML NBs have been implicated in terminal differentiation, growth control (Wang et al., 1998b) and apoptosis (Quignon et al., 1998; Wang et al., 1998a). PML NBs are disrupted in acute promyelocytic leukemia (APL) due to a t(17;15) chromosomal translocation that leads to the formation of heterodimers between wild-type PML protein and a fusion protein composed of the PML protein and the retinoic acid receptor alpha (Dyck et al., 1994). The disruption of the PML NBs is associated with a block in promyelocyte differentiation that can be reversed by treatment with all-trans retinoic acid (Huang et al., 1988; Weiss et al., 1994) or arsenic trioxide (Chen et al., 1996). Both compounds restore PML NBs (Muller et al., 1998) and result in the restoration of normal growth control and the remission of APL (Huang et al., 1988). In the $PML^{-/-}$ transgenic mouse, differentiation of the myelocyte lineage is reduced and terminal differentiation in response to retinoic acid is lost (Wang et al., 1998b).

The composition of PML NBs is altered during the cell cycle and in response to cellular stresses, such as viral infection and heat shock (Sternsdorf et al., 1997). Changes in protein components of PML NBs are associated with changes in modification of the PML protein, primarily phosphorylation and modification by the small ubiquitin-like modifier 1 (SUMO-1) protein (Everett et al., 1999). Targeting of the PML protein to PML NBs in interphase requires modification by SUMO-1 (Muller et al., 1998). SUMO-1 modified PML recruits the proapoptotic protein Daxx to PML NBs (Ishov et al., 1999; Li et al., 2000) resulting in the inhibition of Daxx mediated transcriptional repression (Li et al., 2000) and enhanced Fas meditated cell death (Torii et al., 1999). Likewise, overexpression of ectopic PML results in decreased cell growth in colony forming assays due to increased apoptosis (Fagioli et al., 1998).

The role of AA-PBs in telomere maintenance is unclear. However, given the demonstrated role of the PML NB in the regulation of apoptosis, it has been proposed that AA-PB positive cells may represent a subpopulation of cells that have left the cell cycle and are in the process of dying. To test this hypothesis, we utilized several cell lines derived from human ovarian surface epithelium (HOSE) that are telomerase negative and that utilize the ALT pathway for telomere maintenance. We found that AA-PB positive cells can undergo DNA replication and that mitotic chromosomes contain AA-PBs. These observations suggest that cells containing AA-PBs are competent to progress through the cell cycle, at least in the short term. In addition, we found that the frequency of cells containing AA-PBs increases in the G_2/M phase of the cell cycle and that that the majority of the AA-PB positive cells in the culture are not destined for immediate apoptosis. These data argue against AA-PBs representing a marker of terminal cells that are no longer contributing to the population. Instead, the data raise the possibility that AA-PBs may be involved in the ALT pathway, perhaps representing a clustering of factors required for telomere maintenance via this mechanism.

MATERIALS AND METHODS

Cell lines and culture conditions

HOSE cell lines were derived as described previously (Auersperg et al., 1995). Cells were maintained in a 1:1 mixture of Medium 199 and MCDB-105 medium, supplemented with 4% fetal bovine serum and 0.2 I.U./ml pork insulin (Novagen). Each line was subcultured 1:4 at 80% confluency during the course of these experiments.

Cultures were arrested in S phase by addition of hydroxyurea (Sigma) at 1 mM to the culture medium for 24 hours. The cells were harvested at this time for immunofluorescence and FACS analysis or released from S phase arrest by washing three times with culture medium to remove the hydroxyurea. Released cells were then harvested at various timepoints for FACS and immunofluorescent analysis. Cultures were arrested in G_2/M phase by addition of nocodazole (Sigma) to 1.5 μg/ml for 24 hours and harvested for analysis.

Southern analysis

Genomic DNA was isolated from logarithmically growing cells following standard procedures. Digestion of genomic DNA, quantitation of DNA, agarose gel electrophoresis and Southern transfer were as previously described (Ludérus et al., 1996; van Steensel and de Lange, 1997). 100 μg of each of the telomeric oligonucleotides $(TTAGGG)_4$ and $(CCCTAA)_4$ were end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase and were used as probes to detect telomeric DNA. Filters were hybridized and washed as previously described (Broccoli et al., 1996).

TRAP assay

Whole cell extracts were prepared as described previously (van Steensel and de Lange, 1997). Protein concentrations in extracts were determined using the Bradford assay (Bio-Rad). Telomerase activity was detected using a modification of the TRAP assay (Broccoli et al., 1995; Kim et al., 1994) as described and 0.1 μg to 1 μg of extract. Addition of RNase to the reaction mixture was used to confirm that reaction products resulted from telomerase activity. Addition of 0.5 μg of HeLa extract in reactions containing extracts from telomerase negative cell lines was used to control for reaction conditions and to demonstrate the absence of a diffusible inhibitor of the TRAP reaction in telomerase negative cell lines.

Indirect immunofluorescence and antibodies

Cells were grown directly on coverslips and processed for immunofluorescent analysis as described previously (Chong et al., 1995). For co-localization of the telomeric protein hTRF1 with PML nuclear bodies, cells were fixed in 3.7% formaldehyde (Fisher) in 1× PBS for 10 minutes and permeabilized in 0.5% NP-40 in 1× PBS prior to incubation with antibodies. A rabbit polyclonal antibody against a peptide contained in the amino terminal acidic domain of hTRF1 (#4) has been derived and was used at a dilution of 1:10,000. Detection of the PML nuclear body was carried out using a goat polyclonal antibody raised against a peptide mapping near the amino terminus of human PML (N-19; Santa Cruz) diluted 1:5,000. Primary antibodies were detected with fluoroscein isothiocyanate (FITC)-conjugated or tetramethyl rhodamine isothiocyanate (TRITC)-conjugated donkey anti-rabbit IgG, FITC-conjugated or TRITC-conjugated donkey anti-

mouse IgG and FITC-conjugated donkey anti-goat IgG (Jackson ImmunoResearch). The secondary antibodies did not cross-react.

Detection of BrdU incorporation was carried out using the In Situ Cell Proliferation Kit (Boehringer) following recommendations of the manufacturer. The telomeric protein hTRF1 was detected using affinity purified #4 at 1:100. BrdU was detected using a mouse monoclonal antibody against BrdU provided by the manufacturer (Boehringer) at 1:100. Secondary antibodies were as described above. Apoptotic cells were detected following TUNEL labeling using the ApopTag Direct In Situ Apoptosis Detection Kit (Oncor) following the recommendations of the manufacturer. DNA was stained with 0.2 μg/ml 4,6-diamino-2-phenylindole (DAPI).

Microscopic analysis was carried out using the Eclipse E800 epifluorescent microscope (Nikon) and images captured using an 3 color CCD camera (Optronics; DEI-750 CE) and Scion Image software.

FACS analysis

Cells were collected by trypsinization, washed two times with PBS/2mM EDTA and fixed in 70% ethanol. Cells were stained with propidium iodide (50 μg/ml) and analyzed using a Becton-Dickinson FacsScan and CellQuest software.

RESULTS

Cell lines derived from human ovarian surface epithelium (HOSE) utilize the ALT pathway for telomere maintenance

We have generated several immortal human ovarian surface epithelial (HOSE) cell lines that differ in the pathway used for telomere maintenance (Auersperg et al., 1995). HOSE cell lines were obtained following infection of primary HOSE cells with SV40. In contrast to primary HOSE cells, which undergo less than 10 population doublings before entering replicative senescence, clones derived following infection with SV40 undergo an additional 20-30 population doublings before ceasing proliferation. A subset of the HOSE cell lines have become immortal (HIO) and have undergone over 100 population doublings in culture. The HIO114 cell line became telomerase positive over time in culture (Fig. 1A, lanes 1-5). In contrast, HIO107, HIO117 and HIO118 cell lines remain telomerase negative (Fig. 1A, lanes 6-11 and data not shown). The TRAP assay may be inhibited by excess protein (Broccoli et al., 1995) or by the presence of an inhibitor which can be titrated away upon dilution of some extracts (Piatyszek et al., 1995). Titration using 0.1-1 μg of HIO107, HIO117 and HIO118 extract did not result in detectable telomerase activity (Fig. 1A, lanes 6-9 and data not shown), indicating that the absence of telomerase activity is not due to non-specific inhibition of the TRAP assay. To control for the possibility that a diffusible inhibitor was responsible for the absence of telomerase activity in these cell lines, we mixed 0.5 μg of telomerase positive HeLa cell extract with the HIO extracts prior to carrying out the TRAP assay (Fig. 1A, lanes 10 and 11). HeLa extract telomerase activity was unaffected by mixing with HIO107 negative extract (Fig. 1A, lanes 10 and 12). Identical results were obtained with the HIO117 and HIO118 cell lines (data not shown). These results confirm that the HIO107, HIO117 and HIO118 cell lines are telomerase negative.

Southern analysis of telomere length demonstrates that telomeres in the HIO107 cell line range in size up to >20 kb in length while those in the telomerase positive HIO114 cell line are approximately 4 kb in length (Fig. 1B). Telomere length in the HIO117 and HIO118 cell lines are also extremely long, with the bulk of the telomeric signal located at the limit of mobility of conventional agarose gels (data not shown). The ALT cell lines have telomeric restriction fragments that look very similar to the established pattern for other ALT cell lines in the literature. Together these data are consistent with the HIO107, HIO117 and HIO118 cell lines utilizing the ALT pathway for telomere maintenance.

In keeping with the previously described ALT pathway, the HIO107 cell line contains a subset of cells with several large complexes in which the telomeric proteins hTRF1 and hTRF2 co-localize with the PML nuclear body (Fig. 1C and data not shown). The HIO117 and HIO118 cell lines also contain these structures in a subset of cells (data not shown).

Are AA-PB positive cells cycling?

AA-PBs are present in only a fraction of cells in any given ALT positive cell line. It is possible that AA-PBs may represent an endpoint in these cells and be indicative of non-viable cells. To determine if AA-PB positive cells are traversing the cell cycle we pulse labeled logarithmically growing HIO107 cells with the nucleotide analog BrdU for 30 minutes and then immediately fixed the cells in order to identify cells in S phase. Cells that had incorporated BrdU were detected using a monoclonal antibody against BrdU. At the same time, AA-PB positive cells were detected by staining with an antibody raised against a peptide contained in the amino terminus of hTRF1. AA-PB positive cells that had incorporated BrdU were readily detected (Fig. 2), indicating that such cells are able to carry out DNA replication. The frequency of AA-PB positive/BrdU positive cells was lower than AA-PB negative/BrdU positive cells. On average 30% of AA-PB positive cells had incorporated BrdU while greater than 40% of the AA-PB negative cells incorporated BrdU in the same time period (Table 1).

To determine if characteristics of the HIO107 cell line changed with increased time in culture we compared the percentage of AA-PB positive cells that incorporated BrdU in a logarithmically growing culture during a 30 minute time period in HIO107 cells at different population doublings (Table 1). Despite extensive culture, the percentage of interphase cells that were in S phase was unchanged regardless of the proliferative age of the culture; even after 133 PD approximately 30% of AA-PB positive cells incorporate BrdU. This result suggests that the frequency of cycling cells that contain these multiprotein complexes remains stable over time. Similarly, in the HIO117 and HIO118 cell lines 22% and 32% respectively of AA-PB positive cells incorporated BrdU during a 30 minute time period (Table 1).

Table 1. The frequencies of AA-PB positive versus AA-PB negative cells that incorporate BrdU in a 30 minute time period

Cell line	BrdU+/AA–PB– (%)	BrdU+/AA–PB+ (%)
HIO107, PD 83	35/89 (40)	16/56 (28)
HIO107, PD 133	39/102 (38)	11/39 (28)
HIO117, PD 83	38/55 (69)	4/18 (22)
HIO118, PD 122	34/57 (60)	6/19 (32)

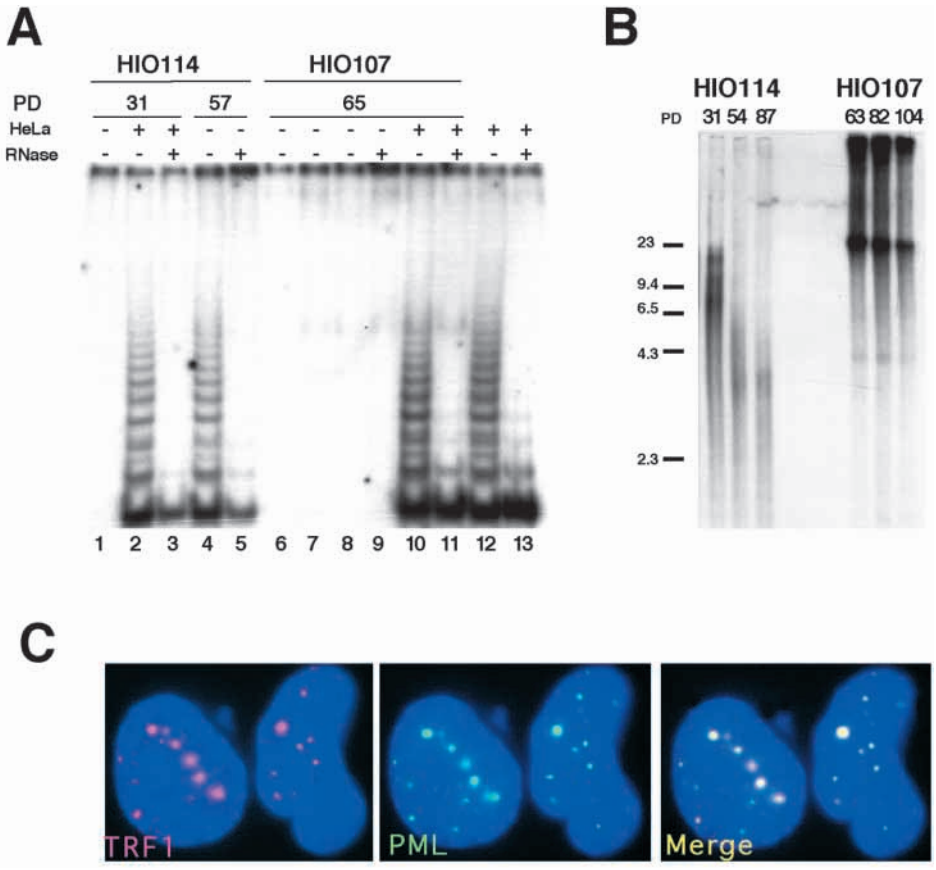


Fig. 1. The HIO114 and HIO107 cell lines use different mechanisms for telomere maintenance. (A) TRAP assay to detect telomerase activity in whole cell extracts prepared from the HIO114 and HIO107 cells at the indicated population doublings (PD). HIO114 upregulated telomerase by PD 57 (lane 4), while the HIO107 cell line remained telomerase negative. As expected, inclusion of RNase in the reaction inhibited formation of telomerase products (lanes 3, 5, 11, 13). Titration of the amount of extract used, from 0.1-1 μg (lane 6, 0.1 μg; lane 7, 0.5 μg; lane 7, 1 μg), does not restore telomerase activity in the HIO107 cell line (lanes 6-9) demonstrating that the TRAP reaction is not inhibited non-specifically. Mixing 0.5 μg of telomerase positive HeLa cell extract with HIO107 extract does not affect the level of telomerase activity (compare lanes 10 and 12), demonstrating the absence of a diffusible inhibitor. (B) Southern blot of telomeric restriction fragments from DNA prepared at the indicated population doublings. The bulk of the telomeric signal in the HIO107 cell line is >20 kb, consistent with this cell line utilizing the ALT pathway for telomere maintenance. No DNA was visible on the gel following staining with ethidium bromide supporting complete digestion. (C) Indirect immunofluorescence of HIO107 cells demonstrating co-localization of the double-stranded telomere DNA binding protein hTRF1 (red) with the PML protein (green) in large subnuclear structures. This phenotype is also consistent with the HIO107 cell line utilizing the ALT pathway. DNA is stained with DAPI (blue).

AA-PB positive cells undergo mitosis

We readily observe large hTRF1 containing structures on cells in metaphase and in anaphase (Fig. 3A), suggesting that AA-PB positive cells can undergo mitosis. To establish that these structures were indeed AA-PBs rather than strong hTRF1 signals associated with extremely long telomeres, logarithmically growing cells were fixed and the presence of AA-PBs determined based on co-localization of the hTRF1 signal with the PML protein. Co-localization of hTRF1 with PML persists on mitotic chromosomes (Fig. 3B). These observations indicate that AA-PB positive cells can enter mitosis but does not address the fate of such cells.

The frequency of AA-PB positive cells is increased in cells in G_2/M phase of the cell cycle

AA-PBs are present in only a subset of cells in ALT cell lines. To determine if the co-localization of telomeric components with the PML NB occurs in cells at a specific stage of the cell cycle we arrested HIO107 and HIO118 cell lines in S phase by exposure to hydroxyurea (HU) and in M phase by exposure to nocodazole. FACS analysis confirmed that both cell lines arrested at the appropriate stage of the cell cycle (Fig. 4A). AA-PB positive cells were identified following indirect immunofluorescence to detect hTRF1. The frequency of AA-PB positive cells was not increased in hydroxyurea-blocked HIO107 or HIO118 cells relative to logarithmically growing cells (Fig. 4B). However, we reproducibly observed a 3- to 4-fold increase in the frequency of AA-PB positive cells following nocodazole arrest (Fig. 4B). AA-PB structures were present at a similar frequency in cells containing both condensed chromosomes and in G_2 cells in the nocodazole treated population. These data suggested that AA-PBs might form in some cells at late S or in the G_2 phase of the cell cycle and be maintained through mitosis.

To exclude the possibility that AA-PBs might have accumulated in these cells as a result of a prolonged period in G_2/M due to the action of nocodazole, we arrested cells in S phase by exposure to HU and determined the frequency of AA-PB positive cells following release from S phase arrest. FACS analysis at several timepoints following release was used to monitor the progression of the culture through the cell cycle (Fig. 5). HIO107 cells are efficiently arrested by HU and then the majority progress through the cell cycle following release by removal of HU. The bulk of the population is in mid-S phase 7 hours after removal of HU and there is a subsequent enrichment in G_2/M cells at 10 and 12 hours post-release. By 14 hours, an increase in the proportion of cells in G_1 phase begins to occur. The frequency of AA-PB positive cells paralleled the enrichment of cells in G_2/M phase (Fig. 5). We reproducibly observed an approximately 2-fold increase in the number of AA-PB positive cells at 10 and 12 hours post release. This result is consistent with AA-PBs forming in the period from late S phase to G_2 phase of the cell cycle. As the cells leave mitosis and re-enter G_1 the number of AA-PB

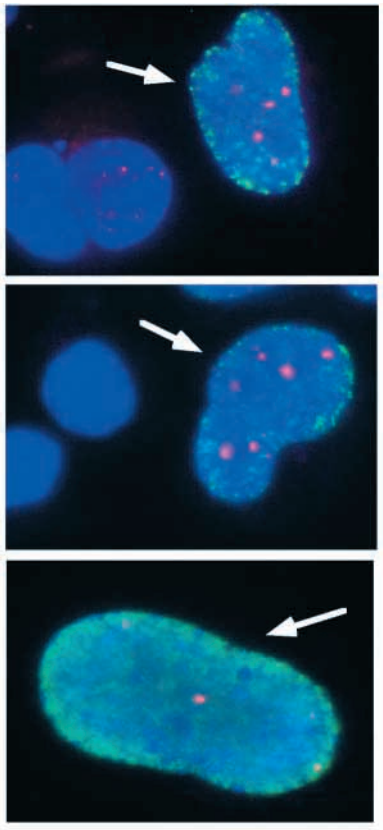


Fig. 2. Cells positive for AA-PBs incorporate BrdU. Indirect immunofluorescence with antibody #4 against hTRF1 was used to identify AA-PB positive cells (red, arrows). Cells in the same field which had incorporated BrdU during a 30 minute pulse just prior to fixation were detected with a mouse monoclonal antibody against BrdU (green). DNA is detected by staining with DAPI (blue).

positive cells decreases, suggesting that the co-localization of telomeric proteins with the PML protein may be a dynamic process. This result is seen in all ALT positive cell lines tested here irrespective of PD.

It is possible that AA-PB positive cells have a limited time in culture. Thus, although we have demonstrated that AA-PB positive cells undergo S phase, can enter mitosis and are increased in frequency in populations enriched for G_2/M phase cells, the cells may initiate an apoptotic program in the subsequent G_1 phase. Such a scenario provides an alternative explanation for the decrease in AA-PB positive cells as the cultures re-enter G_1 (Fig. 5). The enrichment in AA-PB positive cells following synchronization of the culture in S phase by exposure to HU permitted us to address the fate of AA-PB positive cells that have undergone mitosis. Approximately 0.6% of the cells in a logarithmically growing culture of the HIO107 cell line are TUNEL positive, and thus are undergoing apoptosis (Fig. 6). The frequency of TUNEL positive cells is much lower than that of AA-PB positive cells present in the same culture, 19%, suggesting that AA-PB positive cells do not routinely undergo apoptosis in the HIO107 cell line. In addition, the frequency of TUNEL positive cells did not parallel the frequency of AA-PB positive cells in a population arrested in S phase by exposure to HU and for the remaining 36 hours following removal of HU (Fig. 6). As expected, frequency of AA-PB positive cells was elevated 14 hours following the removal of HU (from 19% to 34%) and subsequently decreased at 20 hours and 36 hours to 20% and 17%, respectively. During this time, TUNEL positive cells reached a peak frequency of 2.2% at 20 hours following the removal of HU. Thus although there was a 14% decrease in the frequency of AA-PB positive cells from 14 hours to 20 hours, this was not paralleled by a similar increase in TUNEL positive cells. Similar frequencies of AA-PB positive cells and TUNEL positive cells were observed in two additional experiments (data not shown). In addition, there was no dramatic increase in the percentage of TUNEL positive cells, i.e. less than 2% TUNEL positive, at either 24 or 30 hours following removal of HU (data not shown).

The frequency of AA-PB positive cells is less than would be expected if all the cells in the population formed these structures during late S phase to G_2 phase of the cell cycle. This result raises the possibility that AA-PB positive cells represent a distinct subpopulation within each cell line. Clonal lines derived from each ALT line studied here failed to yield a homogenous population; all clonal lines contained AA-PB positive cells (data not shown). This result is consistent with AA-PBs representing dynamic structures and raise the

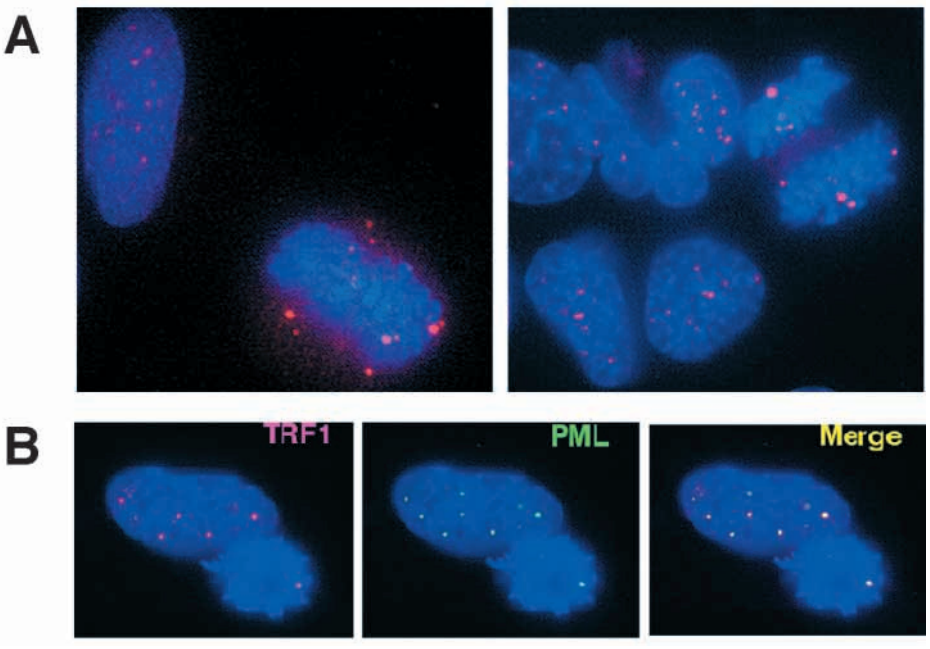


Fig. 3. Cells positive for AA-PBs enter mitosis. **(**A) Examples of an HIO107 cell in either metaphase (left panel) or anaphase (right panel) containing large foci that stain positively with antibody #4 against hTRF1 (red). DNA is detected by staining with DAPI (blue). (B) Co-localization of hTRF1 with the PML protein in a mitotic cell. hTRF1 was detected with antibody #4 (red), PML with antibody N-19 (green) and DNA is detected by staining with DAPI (blue).

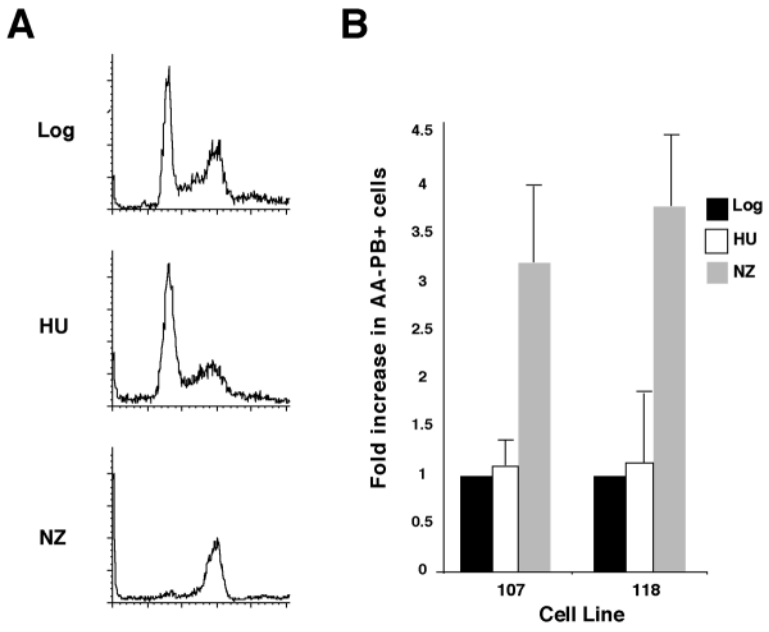


Fig. 4. The frequency of cells that contain AA-PBs is increased in cells arrested in the G_2/M phase of the cell cycle. (A) Representative cell cycle profiles of untreated (Log), hydroxyurea blocked (HU) or nocodazole blocked (NZ) HIO107 cells. X-axis, DNA content; Y-axis, number of events. (B) Quantitation, derived from 3 experiments, of the frequency of either HIO107 or HIO118 cells in the population containing AA-PBs. There is a reproducible enrichment of the number of cells containing AA-PBs of 3- to 4-fold relative to the frequency of AA-PB positive cells in a logarithmically growing culture when cells are blocked in G_2/M phase of the cell cycle by exposure to NZ (*t*-test HIO107, P=0.007; HIO118, P=0.03).

possibility that the formation of AA-PBs might be epigenetically regulated.

DISCUSSION

A novel form of the PML body has been described in cell lines which apparently utilize the ALT pathway for telomere maintenance (Yeager et al., 1999). These structures, called ALT-associated PML bodies (AA-PBs), are unique to telomerase negative cell lines and exhibit co-localization of telomeric DNA and the telomere binding proteins hTRF1 and hTRF2 into multiprotein nuclear structures that also contain the PML protein. In telomerase positive cells, hTRF1 and hTRF2 occupy a distinct nuclear domain from the PML NBs. In addition, AA-PBs correlate with telomere maintenance via the ALT pathway in that they appear in these cultures at a time coordinate with the appearance of the ultra-long telomeric restriction fragments characteristic of the ALT pathway (Yeager et al., 1999).

Although a provocative association, in the absence of further data the role, if any, of AA-PBs in telomerase independent telomere maintenance was unclear. Recently, the PML NBs have been linked to increased sensitivity to Fas meditated apoptosis through recruitment of Daxx to PML NBs by SUMO-1 modification of the PML protein (Li et al., 2000; Torii et al., 1999). In addition, disruption of PML NBs is linked to a block of terminal differentiation in hematopoietic cells (Wang et al., 1998b). Overexpression of PML protein is also correlated with growth suppression in colony forming assays, although growth suppression does not appear to require localization of the PML protein to NBs (Fagioli et al., 1998). Reduced colony formation in these experiments was due to an increased frequency of apoptosis. Based on these studies, it has been suggested that AA-PBs may only be present in cells that are marked for death and thus may represent a terminal phenotype.

To test this hypothesis we carried out a series of experiments aimed at addressing the viability of AA-PB positive cells. We demonstrate that AA-PBs may be present in mitotic cells. In addition, cells containing these structures incorporate BrdU and the frequency of cells containing AA-PBs that incorporate BrdU is constant over prolonged time in culture. These observations are consistent with the interpretation that AA-PB positive cells are viable and argue against this phenotype being associated with immediate cell death. Finally, we demonstrate that an experimentally induced increase in the frequency of AA-PB positive cells in a synchronized culture is not followed by a subsequent and equivalent increase in the number of apoptotic cells. Thus, association of telomeric components with the PML NB does not a priori indicate a cell destined for cell cycle arrest or apoptosis. Although the experiments carried out here do not address the long-term fate of AA-PB positive cells, our data support the conclusion that these cells are viable components of cell lines that utilize the ALT pathway for telomere maintenance. Attempts to directly monitor the fate of these cells using live imaging techniques have proved inconclusive; fewer than 60% of the cells underwent mitosis within the 56 hour time course of the experiment. Since the frequency of AA-PB positive cells is on average 20% of the population, this indicated that many of the AA-PB negative cells had not divided during this period. If we had observed greater than 95% of the cells undergoing mitosis this would have provided support, albeit indirect, that at least some AA-PB positive cells are dividing.

We demonstrate that the frequency of cells containing AA-PBs increases in a population enriched for cells in the G_2/M phase of the cell cycle. This result is consistent with the formation of AA-PBs during a period of the cell cycle from late S phase to G_2/M phase. Furthermore, the decreased frequency of AA-PBs that is observed as the cells exit mitosis and re-enter the G_1 phase of the cell cycle suggests that the co-localization of telomeric proteins with PML NBs is a dynamic process. Consistent with these observations, it has recently been shown that the gene product of Nijmegen breakage syndrome, NBS1, is localized to AA-PBs in late S to G_2 phase

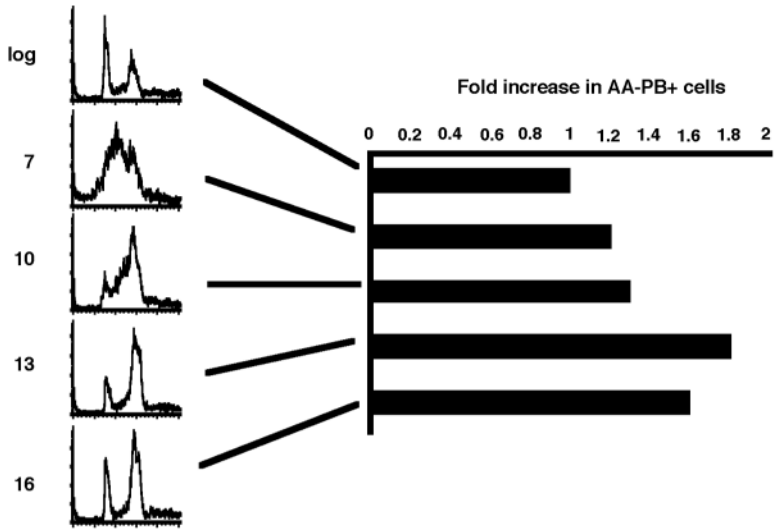
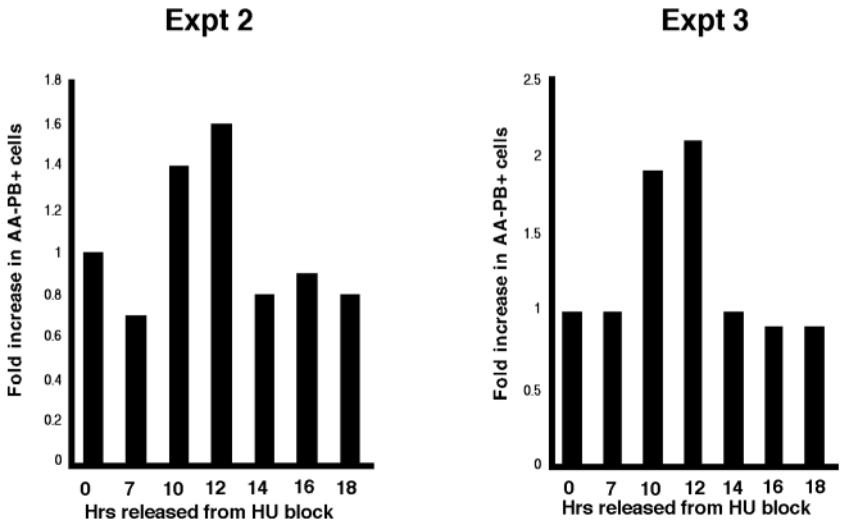


Fig. 5. The frequency of HIO107 cells containing AA-PBs is increased as a synchronized population enters the G_2 phase of the cell cycle. Representative cell cycle profile demonstrating HU arrest and synchronous progression through the cell cycle. Time after the removal of HU is indicated to the left of each profile. Quantitation of the frequency of HIO107 cells that contained AA-PBs in untreated cells (Log) and at the indicated times after release from the HU block. Results from two additional experiments are shown below. The frequency of AA-PB positive cells is expressed as a fold-increase following normalization to the frequency of AA-PB positive cells in a logarithmically growing culture.

of the cell cycle (Wu et al., 2000). The HOSE cell lines used here are refractory to arrest with agents that act in G_1 phase of the cell cycle and are not sensitive to reduced serum or contact inhibition. Thus, we were unable to directly ascertain if association of hTRF1 with the PML protein is reduced in a population of cells enriched for the G_1 phase of the cell cycle.

It has previously been reported that the PML protein is deconjugated from SUMO-1 during mitosis and that this alteration in protein modification is correlated with an altered association of PML NB associated proteins, such as Sp100 and Daxx (Ishov et al., 1999). Here we demonstrate that co-localization of TRF1 and the PML protein persists in mitotic cells. This observation suggests that SUMO-1 modification of the PML protein may not be essential for the localization of telomeric proteins to PML NBs. The co-localization of telomeric components with the PML protein in mitosis represents a novel co-localization for this protein.



The HOSE cell lines utilized for these studies were all immortalized following infection with SV40, raising the possibility that the phenotypes observed here are unique to SV40. This possibility is unlikely because it has previously been demonstrated that utilization of the ALT pathway is not determined by the method of immortalization. In addition, the cell lines utilized for these studies are all derived from human ovarian surface epithelium. Thus, it is possible that some of the phenotypes observed here may be specific to cells of epithelial origin. Future studies comparing the response of ALT cell lines derived from different tissues and following different immortalization schemes will establish if the phenotypes observed here are common to the ALT pathway or define a different mechanism of telomerase independent telomere stabilization.

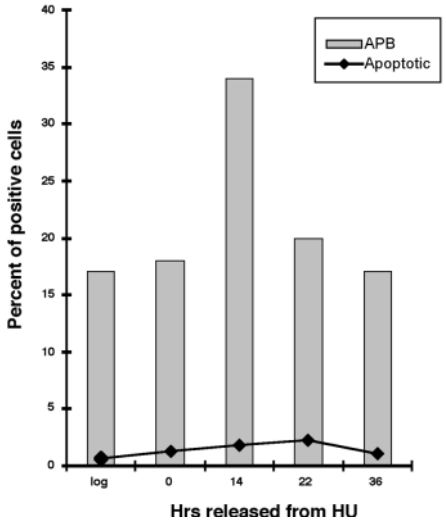


Fig. 6. The majority of AA-PB positive cells do not undergo apoptosis. Number of positive cells (Y-axis) plotted against hours following removal of HU (log, logarithmically growing cells and 0, HU arrested cells). Although a slight increase in the frequency of TUNEL positive cells (2.2%) is observed 22 hours after the removal of HU, this is not sufficient to account for the 14% decrease in the frequency of AA-PB positive cells observed as the culture progresses from a mitotically enriched population (14 hours) to the G_1 phase (22 hours).

The results presented here argue against AA-PBs being associated only with cells that are terminally differentiated or are no longer contributing to culture viability. The data also support the hypothesis that these structures are, at least in part, coordinately regulated with the cell cycle. The presence of AA-PBs in clonal cell lines derived from three independent ALT cell lines raises the possibility that the formation of these structures may be regulated. For example, these structures may form in cells with critically short telomeres. However, it is also possible that AA-PBs are storage facilities rather than structures that play a direct role in telomere maintenance and further experiments are required to address this issue. Given our data indicating that AA-PB positive cells are viable, it becomes critically important to determine if these structures are actively involved in telomere maintenance via the ALT pathway. Future experiments aimed at manipulation of the AA-PB structures will determine if these novel nuclear bodies are instrumental in telomerase independent telomere maintenance via the ALT pathway.

Telomerase activity is the most prevalent mechanism used by tumors to circumvent the telomere length dependent check on proliferation. Forced expression of telomerase is sufficient to generate the immortal phenotype required for tumor formation. In addition, inhibition of telomerase has been shown to limit the growth of transformed cells in vitro. Based on these observations, inhibition of telomerase has become an attractive modality for the development of future chemotherapeutic agents. However, a proportion of tumors are telomerase negative. These tumors would be refractory to treatment with telomerase inhibitors. Thus, understanding the mechanism(s) responsible for telomerase independent telomere maintenance would allow development of strategies to combat growth of this class of tumors.

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