Telomere Instability in a Human Tumor Cell Line Expressing NBS1 With Mutations at Sites Phosphorylated by ATM

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Abstract
Nijmegen breakage syndrome (NBS) is an autosomal recessive disease demonstrating a wide range of phenotypic abnormalities, including premature aging, increased cancer incidence, chromosome instability, and sensitivity to ionizing radiation. The gene involved in NBS, NBS1, is part of the MRN complex (MRE11/RAD50/NBS1 (MRN)) complex that also includes MRE11 and RAD50, which is involved in DNA repair and cell cycle regulation in response to DNA damage. The MRN complex is also involved in telomere maintenance, as demonstrated by the shortened telomeres in NBS primary human fibroblasts and the association of NBS1 with the telomere-binding protein TRF2. To learn more about how a deficiency in telomere maintenance might contribute to chromosome instability in NBS, we have investigated the stability of telomeres in two telomerase-positive human tumor cell lines, BNmt-On and BNmt-Off, expressing an inducible NBS1S278A/S343A gene containing mutations at serines 278 and 343 phosphorylated by ATM. The results demonstrate an increased rate of telomere loss in both clones following expression of NBS1S278A/S343A. The absence of detectable changes in average telomere length suggests that NBS1-associated telomere loss results from stochastic events involving complete telomere loss or loss of telomere capping function. The recombination events associated with telomere loss were found to be similar to those shown previously to result in breakage/fusion/bridge cycles, suggesting that telomere loss can contribute to chromosome instability in NBS1-deficient cells. Telomere loss showed no correlation with radiosensitivity or radiosensitive DNA synthesis, demonstrating that NBS1S278A/S343A promotes telomere loss through a separate pathway from these other phenotypes associated with NBS.

Introduction
Nijmegen breakage syndrome (NBS) is an autosomal recessive disease demonstrating a wide range of phenotypic abnormalities, including premature aging, increased cancer incidence, chromosome instability, and sensitivity to ionizing radiation (1, 2). NBS results from mutations within the NBS1 gene, which is part of the MRN complex that also contains MRE11 and RAD50 proteins (3, 4). The MRN complex is activated in response to double-strand breaks (DSBs), which requires phosphorylation of NBS1 by the ATM protein involved in ataxia-telangiectasia (AT; 5–7). This activation of the MRN complex by ATM is required for proper cell cycle regulation and DNA repair, as demonstrated by the similarities in the abnormalities associated with NBS and AT (2) as well as the AT-like disease involving mutations in MRE11 (8). As a result, mammalian cells deficient in NBS1 lack the S-phase cell cycle checkpoint (9) and increased chromosome damage in response to ionizing radiation (10). However, the exact nature of the repair defect in NBS is not completely understood. The MRN complex in yeast, which contains the orthologue of NBS1, XRS2, is involved in repair of DSBs by homologous recombination but not by NHEJ (11) and is required for NHEJ of DSBs containing hairpins at their ends (12). In contrast to yeast, the MRN complex in chicken DT40 cells is involved in repair of DSBs by homologous recombination but not by NHEJ (13, 14). The MRN complex is also involved in repair of DSBs in mammalian cells, although most DSBs are repaired in AT (15, 16) and NBS (17, 18) cells. Evidence for a role in repair of DSBs is shown by the association of MRN with DSBs in human cells (19, 20) and the fact that MRN foci are not observed in NBS cells after ionizing radiation (3). NBS1 is also associated with BRCA1 (21, 22) and the phosphorylated form of histone H2AX (23), both of which are involved in DSB repair.

The MRN complex also functions in telomere maintenance. Telomeres are composed of a 6-bp repeat sequence and associated proteins that form a cap that protects chromosome ends (24, 25). Telomeric repeat sequences are added on by the enzyme telomerase, which compensates for the loss of DNA from the ends of chromosomes during cell division. In humans, telomerase is active in germ line cells but inactive in most somatic cells (26). As a result, telomeres become progressively shorter in somatic cells, which has been proposed to limit cellular life span by promoting replicative cell senescence (27). Consistent with this hypothesis, transfection of human fibroblasts with telomerase greatly extends their life span (28). A primary function of telomeres is to prevent chromosome fusion, as illustrated by the large increase in chromosome fusion observed in somatic cells that fail to senesce and therefore continue to undergo telomere shortening (29). Increased chromosome fusion is also seen in cells with...
mutations in genes that affect telomere function, including DNA-Pkcs, Ku, and TRF2 (30–34). The ability of cells to properly maintain telomeres is therefore a critical factor in maintaining chromosome stability.

Evidence for a role of the MRX complex in telomere maintenance is seen in yeast, where defects in the MRX complex result in shortened but stable telomeres (35, 36). In this respect, mutations in any of the MRX proteins are similar to mutations in TEL1, which also result in short but stable telomeres (37). Because mutations in either the MRX complex or TEL1 result in continuous shortening of telomeres when combined with mutations in MEC1 (38, 39), MRX and TEL1 appear to work in the same pathway. Similarly, NBS primary fibroblasts also have shortened telomeres, which has been proposed to have a role in the pathology of this disease (40). In this respect, NBS fibroblasts are similar to AT fibroblasts, which also show accelerated telomere shortening (41). In fact, mice with combined knockouts in both ATM and the RNA component of telomerase show accelerated telomere loss and premature aging, leading to the hypothesis that telomere loss is the reason for some of the phenotypic abnormalities observed in AT (42). Further evidence for a role of the MRN complex in telomere maintenance is found in its association with telomeres and interaction with TRF2 (43). MRE11 and RAD50 are found at the telomere throughout the cell cycle, while NBS1 is associated with the telomere only during S phase, suggesting that NBS1 is required for telomere replication. Thus, like TRF2 (44, 45), NBS1 may be required for the formation and/or function of the single-stranded tails required to cap the ends of chromosomes.

We investigated previously the consequences of spontaneous telomere loss in clone B3 of the EJ-30 human tumor cell line, which has a herpes simplex virus thymidine kinase (HSV-tk) selectable marker gene integrated immediately adjacent to a telomere (46, 47). Loss of the HSV-tk gene was found to be associated with the formation of inverted repeats, large duplications, and prolonged periods of instability specific to the “marker” chromosome containing the telomeric HSV-tk gene. The absence of a telomere and the presence of anaphase bridges involving the marker chromosome more than 20 generations after the loss of the telomere demonstrated that this chromosome instability involved breakage/fusion/bridge (B/F/B) cycles (47). In the present study, the influence of NBS1 on telomere function was investigated using two clones of B3, BNmt-On and BNmt-Off, which contain a mutant NBS1 gene, NBS1S278A/S343A, under the control of tetracycline-inducible promoters. NBS1S278A/S343A contains mutations at serines 278 and 343 that are sites of phosphorylation by ATM in response to DNA damage (5). Cultures of BNmt-On and BNmt-Off with and without expression of NBS1S278A/S343A were analyzed for the frequency of telomere loss, the rate of loss of the telomeric HSV-tk gene, and the types of rearrangements associated with telomere loss. For comparison with other phenotypes associated with NBS, BNmt-On and BNmt-Off were also analyzed for changes in the rate of DNA synthesis and cell survival in response to ionizing radiation.

Results

Development of Cell Lines

The role of NBS1 in telomere loss and chromosome instability in human cells was investigated using clone B3 of the human EJ-30 bladder cell carcinoma cell line (46, 47). Clone B3 contains a selectable HSV-tk gene located

![FIGURE 1. Structure of the plasmid sequences integrated adjacent to a telomere in clone B3 of the EJ-30 tumor cell line. The pNCT-tel plasmid linearized with NotI was used to seed the formation of a new telomere on the end of chromosome 16p. The location of the cellular DNA, plasmid vector (amp/ori), neo gene, HSV-tk gene, and telomeric repeat sequences are shown. The location of BamHI (Bm) and ClaI (Cl) restriction sites used for Southern blot analysis and the sizes of the restriction fragments are also shown.](mcr.aacrjournals.org)
immediately adjacent to a telomere on chromosome 16 (Fig. 1). Clone B3 was established by transfection with the linearized pNCT-tel plasmid that contains telomeric repeat sequences on one end. The integration of a plasmid containing telomeric repeat sequences on one end results in the “seeding” of a new telomere on the end of a broken chromosome (48–50). The seeded telomeres are elongated in culture, and their length and dynamics become similar to the other telomeres in the cell (51, 52). Selection with ganciclovir for cells that lose the HSV-tk gene provides a method for determining the rate of telomere loss and for the analysis of the types of chromosome rearrangements that occur (46, 47, 53).

To investigate the influence of NBS1 on telomere stability, we established clones of B3 that contain the NBS1S278A/S343A gene, which has mutations in serines 278 and 343 that are sites of phosphorylation by ATM in response to DNA DSBs (5). The NBS1S278A/S343A gene in these clones is under the control of a tetracycline-responsive promoter. Two different tetracycline-responsive systems were used. In clone BNmt-On, NBS1S278A/S343A is regulated using the RetroTet ART tetracycline-inducible system (54). This system uses a promoter that responds to an activator that is active in the presence of tetracycline or doxycycline and a repressor that is inactive in the presence of tetracycline or doxycycline. Inducible expression of NBS1S278A/S343A in clone BNmt-On was confirmed by reverse transcription-PCR (RT-PCR) using primers that are specific for the viral construct and therefore do not amplify endogenous NBS1 mRNA. BNmt-On showed expression of NBS1S278A/S343A following growth for 2 weeks in the presence of doxycycline (Fig. 2, A and B) but no detectable expression in the absence of doxycycline. A clone of B3, BNwt-On, containing the wild-type NBS1 gene under the control of the RetroTet ART system also shows inducible expression, with no detectable expression in the absence of doxycycline (Fig. 2C). Clone BNmt-Off contains the Tet-Off system that uses an activator that is inactive in the presence of tetracycline or doxycycline (55). Thus, in contrast to BNmt-On, BNmt-Off expresses NBS1S278A/S343A in the absence of telomeric or doxycycline. Inducible expression of NBS1S278A/S343A in clone BNmt-Off was also confirmed by RT-PCR (Fig. 2D). BNmt-Off showed no expression of the transduced NBS1S278A/S343A with doxycycline present, while expression of NBS1S278A/S343A was detectable following growth for 2 weeks in the absence of doxycycline.

Telomere Instability and NBS1

The influence of NBS1S278A/S343A expression on telomere stability in BNmt-On and BNmt-Off was investigated by fluorescence in situ hybridization (FISH) using a telomere-specific peptide nucleic acid (PNA) probe. Detection of telomeres with PNA probes is highly sensitive and can detect telomeres as small as 150 bp (56). As a result, telomeres can be detected on nearly all chromosomes in metaphase spreads in the absence of expression of NBS1S278A/S343A (Fig. 3A). However, in cells expressing NBS1S278A/S343A, we noted an increase in the frequency of chromosomes without detectable telomeres on one (Fig. 3B) or both (Fig. 3C) chromatids. Cells expressing NBS1S278A/S343A also showed an increased frequency of dicentric chromosomes (Fig. 3D). Cultures of BNmt-Off grown in medium containing doxycycline showed relatively few dicentric chromosomes (1 in 200 metaphase spreads), while BNmt-Off cultures grown in medium without doxycycline for more than 2 weeks showed increased dicentric chromosomes (10 in 200 metaphase spreads).

To further investigate the influence of NBS1S278A/S343A on telomere loss, the frequency of chromosomes without detectable telomeres was investigated at various times after expression of NBS1S278A/S343A. In clone BNmt-On, the frequency of chromosomes without detectable telomeres increased with time after addition of doxycycline, eventually reaching an average of 1.3 undetectable telomeres/cell after 4 weeks (Fig. 4A). In contrast, BNmt-On cultures grown without doxycycline showed no increase in chromosomes without telomeres during this same time period. Clone BNwt-On, containing the wild-type NBS1 gene, also showed no increase in the frequency of chromosomes without detectable telomeres following expression of NBS1S278A/S343A in cultures grown in medium with doxycycline (Fig. 4A).
As with clone BNmt-On, clone BNmt-Off also showed a continual increase in the number of chromosomes without detectable telomeres following the expression of \(NBS1^{S278A/S343A}\) (Fig. 4B). After 4 weeks without doxycycline, the frequency of undetectable telomeres in BNmt-Off increased to an average of 1 per cell. In contrast, BNmt-Off cultures grown with doxycycline showed no increase in chromosomes without telomeres during this same time period. The combined results demonstrate that the increased rate of telomere loss in BNmt-Off cultures grown without doxycycline is directly related to expression of \(NBS1^{S278A/S343A}\).

The rate of recovery of telomeres in BNmt-On cultures grown previously with doxycycline was investigated following the removal of doxycycline from the medium (Fig. 5A). BNmt-On cultures switched back to the medium without doxycycline showed a reduction in the number of chromosomes without telomeres from 0.8 to 0.1 per cell after 4 weeks. During this same period, the cultures grown in the presence of doxycycline continued to show an increase in the number of chromosomes without detectable telomeres. As with clone BNmt-On, the rate of recovery of telomeres in BNmt-Off cultures grown previously without doxycycline was investigated following the readdition of doxycycline to the medium (Fig. 5B). BNmt-Off cultures switched back to the medium with doxycycline showed a reduction in the number of chromosomes without telomeres from 0.5 to 0.05 per cell after 4 weeks. During this same period, the cultures grown without doxycycline continued to show an increase in the number of chromosomes without detectable telomeres.

**FIGURE 3.** Analysis of telomere loss in clone BNmt-On cultures expressing \(NBS1^{S278A/S343A}\). Cultures of BNmt-On grown either without (A) or with doxycycline (B–D) were analyzed by FISH using a telomere-specific PNA probe. Chromosomes were counterstained with 4',6-diamidino-2-phenylindole. Cells not expressing \(NBS1^{S278A/S343A}\) had telomeres on the ends of most chromosomes (A), while cells expressing \(NBS1^{S278A/S343A}\) showed an increase in the number of chromosomes without detectable telomere on one (B) or both sister chromatids (C, arrows) as well as dicentric chromosomes (D, containing two centromeres (arrows)).
Consequences of Telomere Loss

To investigate the consequences of telomere loss resulting from the expression of \textit{NBS1}^{S278A/S343A}, we studied its influence on the rate of loss of the \textit{HSV-tk} gene located at the telomere on the short arm of chromosome 16. Clones of EJ-30 have a relatively high spontaneous rate of loss of the \textit{HSV-tk} gene \((10^{-3} \text{ events/cell/generation})\) dememtrating that EJ-30 is similar to many other human tumor cells that have telomere instability \((52, 57)\). Fluctuation analysis was performed to determine whether expression of \textit{NBS1}^{S278A/S343A} causes a further increase in the rate of telomere loss. For these studies, small populations of BNmt-On or BNmt-Off were expanded with and without doxycycline and then monitored the frequency of cells in the population that were resistant to both ganciclovir and G418 \((46)\). Digestion of clone B3 and G418 (G418/gan^r). Selection with G418 was used simultaneously with ganciclovir to insure that the neo gene was retained, because some portion of the plasmid is required for subsequent analysis of the types of recombination events involved. Consistent with the cytogenetic analysis, the BNmt-On cells grown with doxycycline demonstrated a 10-fold increase in the rate of G418/gan^r cells as compared with the cells grown without doxycycline (Table 1A). Similarly, the BNmt-Off cells grown without doxycycline \((i.e., \textit{NBS1}^{S278A/S343A})\) demonstrated a 5-fold increase in the rate of G418/gan^r cells as compared with the cells grown with doxycycline (Table 1B). The actual rate of telomere loss is even higher, because G418/gan^r cells represent only those events in which telomere loss occurred without the loss of the neo gene, which represent only a small fraction of the total \((46)\). Thus, in view of the already high rate of spontaneous loss of telomeres in the EJ-30 tumor cell line \((46)\), expression of \textit{NBS1}^{S278A/S343A} results in a large increase in the rate of loss of the \textit{HSV-tk} gene.

Southern blot analysis was performed to investigate the nature of the rearrangements involved in the loss of function of the \textit{HSV-tk} gene in the G418/gan^r subclones (Fig. 6). Loss of the HSV-tk gene in B3 \((46)\), like mouse ES cells \((47, 53)\), was found previously to result in either addition of a telomere at a new location or sister chromatid fusion followed by prolonged B/F/B cycles. The telomeric pNCT-tel plasmid contains a single \textit{BamHI} site in the center of the plasmid (see Fig. 1). Therefore, as reported previously \((46)\), digestion of the unrearranged genomic DNA with \textit{BamHI} produces two bands: a 4.5-kb band that contains cellular DNA and the internal portion of the plasmid and a larger band that contains the terminal restriction fragment that is heterogeneous in length due to the variability in the length of the telomere in different cells in the population \((Fig. 6A)\). Digestion of the unrearranged genomic DNA with both \textit{BamHI} and \textit{ClaI}, which cuts off the telomeric repeat sequences (see Fig. 1), produces the 4.5-kb internal band and a 4.0-kb band containing the \textit{HSV-tk} gene \((Fig. 6B)\). G418/gan^r subclones that have a telomere added at a new location in the \textit{HSV-tk} gene show no detectable change following digestion with \textit{BamHI} alone \((46)\). However, with \textit{BamHI} and \textit{ClaI}, the 4.0-kb band is missing as a result of the loss of the \textit{ClaI} site adjacent to the telomere, and a heterogeneous band appears due to the presence of telomeric repeat sequences in this fragment. G418/gan^r subclones that contain an inverted repeat involving the \textit{HSV-tk} gene due to sister chromatid fusion are missing the heterogeneous band with \textit{BamHI} alone \((46)\). However, with \textit{BamHI} and \textit{ClaI}, the 4.0-kb band is missing as a result of the loss of the \textit{ClaI} site adjacent to the telomere, and a heterogeneous band appears due to the presence of telomeric repeat sequences in this fragment. G418/gan^r subclones that contain an inverted repeat involving the \textit{HSV-tk} gene due to sister chromatid fusion are missing the heterogeneous band with \textit{BamHI} alone and with \textit{BamHI} and \textit{ClaI} and instead show a new band that differs in length in different subclones \((46)\). By these criteria, all five of the G418/gan^r subclones isolated from BNmt-On have rearrangements consistent with sister chromatid fusions. The results therefore suggest that telomere loss resulting from NBS1 deficiency results primarily in chromosome fusion, as was reported previously for spontaneous telomere loss in clone B3 \((46, 47)\).

Analysis of Telomerase Activity and Average Telomere Length in Cells Expressing \textit{NBS1}^{S278A/S343A}

Visual inspection of metaphase spreads from BNmt-On and BNmt-Off hybridized with the telomere-specific PNA probe
indicated no obvious changes in signal intensity on most chromosomes in cells expressing \( \text{NBS1}^{\text{S278A/S343A}} \) (data not shown). Consistent with this observation, no difference in average telomere length was detectable by Southern blot analysis in clone BNmt-Off after growth for 2 or 6 weeks with or without doxycycline (Fig. 7). As might be expected from these results, BNmt-On cultures grown for 4 weeks in the absence of doxycycline showed little or no change in relative telomerase activity compared with BNmt-On cultures grown in the presence of doxycycline (data not shown). Thus, the inability of telomerase to maintain telomere length cannot explain the increased rate of telomere loss in cells expressing \( \text{NBS1}^{\text{S278A/S343A}} \).

**Influence of Ionizing Radiation on Cells Expressing \( \text{NBS1}^{\text{S278A/S343A}} \)**

In view of the results demonstrating that expression of \( \text{NBS1}^{\text{S278A/S343A}} \) promotes telomere loss, we investigated the influence of \( \text{NBS1}^{\text{S278A/S343A}} \) on the rate of DNA synthesis and cell survival following exposure to ionizing radiation. Defects in \( \text{NBS1} \) are known to result in both increased radiosensitivity and loss of the S-phase checkpoint, as measured by a failure to reduce the rate of DNA synthesis in response to ionizing radiation, known as radioresistant DNA synthesis (1, 2). \( \text{NBS1}^{\text{S278A/S343A}} \) expression in clone BNmt-On and wild-type \( \text{NBS1} \) expression in BNwt-On after growth for 6 days in medium containing doxycycline had no effect on the rate of DNA synthesis following exposure to ionizing radiation (Fig. 8A). Similarly, \( \text{NBS1}^{\text{S278A/S343A}} \) expression after growth of clone BNmt-Off for 6 days in medium without doxycycline also had no effect on the rate of DNA synthesis following exposure to ionizing radiation (Fig. 8A). Thus, expression of \( \text{NBS1}^{\text{S278A/S343A}} \) did not result in radioresistant DNA synthesis typical of NBS cells due to the loss of the S-phase checkpoint.

**Table 1. Influence of \( \text{NBS1}^{\text{S278A/S343A}} \) on the Rate of Telomere Loss**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>+Dox</th>
<th>–Dox</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) BNmt-On</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
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<td>3</td>
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<td>4</td>
<td>4</td>
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<tr>
<td></td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Av. no. cells/culture</td>
<td>2.6 x 10^6</td>
<td>6.0 x 10^6</td>
</tr>
<tr>
<td>Rateb</td>
<td>3.2 x 10^{-3}</td>
<td>3.1 x 10^{-3}</td>
</tr>
<tr>
<td>Frequency</td>
<td>2.0 x 10^{-4}</td>
<td>1.4 x 10^{-3}</td>
</tr>
<tr>
<td>Av.</td>
<td>19.8</td>
<td>1.4</td>
</tr>
<tr>
<td>(B) BNmt-Off</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture</td>
<td>1</td>
<td>1</td>
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<td></td>
<td>2</td>
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<tr>
<td>Av. no. cells/culture</td>
<td>3.5 x 10^6</td>
<td>5.3 x 10^6</td>
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<tr>
<td>Rateb</td>
<td>4.8 x 10^{-6}</td>
<td>2.6 x 10^{-6}</td>
</tr>
<tr>
<td>Frequency</td>
<td>2.1 x 10^{-5}</td>
<td>1.7 x 10^{-4}</td>
</tr>
<tr>
<td>Av.</td>
<td>25</td>
<td>17</td>
</tr>
</tbody>
</table>

\( ^{a} \text{Per 100-mm dish containing} \times 10^5 \text{ cells/plate.} \)

\( ^{b} \text{Determined from the equation} \ Cr = \frac{CaNt}{Nt} \text{, where} C \text{ is the number of cultures,} r \text{ is the total number of mutant colonies in the population (no. colonies x total cells /} \times 10^5 \text{),} N_t \text{ is the average number of cells in each of the cultures at the time of plating, and} a \text{ is the rate.} \)
We next investigated the influence of NBS1<sup>S278A/S343A</sup> expression on radiosensitivity in clones BNmt-On and BNmt-Off using clonogenic survival (Fig. 8B). Cultures of BNmt-On expressing NBS1<sup>S278A/S343A</sup> after growth in medium containing doxycycline for 2 weeks showed no increase in radiosensitivity. In contrast, cultures of BNmt-Off expressing NBS1<sup>S278A/S343A</sup> after growth in the absence of doxycycline for 2 weeks showed a substantial increase in radiosensitivity. Thus, clones BNmt-On and BNmt-Off showed different responses to doxycycline and doxycycline withdrawal, indicating the complexity of the telomere function in radiosensitivity.

**FIGURE 7.** The influence of NBS1<sup>S278A/S343A</sup> expression on average telomere length. Genomic DNA from clone BNmt-On grown with (+) or without (−) doxycycline for 3 or 7 weeks and clone BNmt-Off grown with (+) or without (−) doxycycline for 4 or 6 weeks was digested with RsaI and HinfI, and Southern blot analysis was performed using hybridization with a telomeric repeat-specific probe. Molecular size markers consisting of lambda bacteriophage HindIII restriction fragments are shown.

**FIGURE 8.** The effect of NBS1<sup>S278A/S343A</sup> expression on the cellular response to ionizing radiation. A. The rate of DNA synthesis in cultures of BNmt-On, BNmt-On, and BNmt-Off grown with (●) or without (■) doxycycline after exposure to various doses of ionizing radiation. B. Cell survival in cultures of BNmt-On and BNmt-Off grown with (●) or without (■) doxycycline after exposure to various doses of ionizing radiation.
Discussion

The data presented here demonstrate that expression of NBS1<sup>S278A/S343A</sup> results in an increased rate of telomere loss, as was evident from both FISH analysis with a telomere-specific PNA probe and analysis of the rate of loss of a telomeric HSV-<i>tk</i> gene. Telomere loss in cells expressing NBS1<sup>S278A/S343A</sup> appears to involve a stochastic process, because no change in average telomere length was evident in cells expressing NBS1<sup>S278A/S343A</sup> by either FISH or Southern blot analysis. After 28 days, cells expressing NBS1<sup>S278A/S343A</sup> showed an average of 1 telomere loss/cell, demonstrating a rate of loss of 0.035 telomeres/cell/generation, assuming an approximate cell cycle time of 24 h. These results demonstrate that phosphorylation of NBS1 by ATM is important for proper telomere maintenance. NBS1<sup>S278A/S343A</sup> could promote telomere loss through its ability to act as a dominant negative for telomere maintenance by interfering with the proper function of the endogenous NBS1 protein. However, NBS1<sup>S278A/S343A</sup> could also function as a hypomorph, interfering with telomere function due to increased levels of protein with wild-type function. Although overexpression of wild-type NBS1 did not result in telomere loss, the mutant form of the protein could have increased wild-type activity or increased half-life.

Unlike the effects on telomere function, expression of NBS1<sup>S278A/S343A</sup> in clones BNmt-Off and BNmt-On did not result in radioresistant DNA synthesis, although BNmt-Off did show an increase in radiosensitivity. Why there should be a difference in the response in BNmt-On and BNmt-Off with respect to radiosensitivity is unclear, because both were isolated from the same parental cell line. Some studies have found that phosphorylation of NBS1 by ATM at serines 278 and/or 343 is important in radiosensitivity (5, 7, 58), although one study found that phosphorylation at these sites was not important for radiosensitivity (59). Thus, the response of cells to mutations at these phosphorylation sites in NBS1 with respect to radiosensitivity may be cell type dependent. Our results also show that expression of NBS1<sup>S278A/S343A</sup> can result in changes in radiosensitivity without influencing radioresistant DNA synthesis, consistent with earlier results demonstrating that these two endpoints occur through separate pathways (59). Regardless, the fact that NBS1<sup>S278A/S343A</sup> expression results in an increased rate of telomere loss in both BNmt-On and BNmt-Off, although neither clone shows radioresistant DNA synthesis and BNmt-On shows no change in radiosensitivity, demonstrates that the role of NBS1 in telomere maintenance is distinct from its roles in radiosensitivity or regulation of the S-phase checkpoint.

A previous study found shortened telomeres in NBS primary fibroblasts, which could be corrected by transfection with NBS1<sup>S278A/S343A</sup>. However, this earlier study also found that telomere shortening in NBS cells could be corrected by expression of hTERT, the catalytic subunit of telomerase. Thus, the expression of telomerase can compensate for telomere shortening in NBS cells. Consistent with this observation, another study found that telomeres in AT fibroblasts also shorten at an accelerated rate, although the initial telomere length does not differ significantly from that observed in normal cells (41). Thus, increased telomere shortening in AT also appears to occur only in somatic cells that do not express telomerase. Consistent with this possibility, we demonstrated previously that the telomere lengths in telomerase-positive immortal AT cell lines did not differ significantly from that seen in telomerase-positive immortal normal cell lines (60). Therefore, like AT fibroblasts, shortened telomeres in NBS fibroblasts are likely to result from accelerated shortening, while the cells in the present study can compensate for this additional telomere loss due to the expression of telomerase. However, even with the expression of telomerase, in cells expressing NBS1<sup>S278A/S343A</sup>, stochastic events can occur that result in loss of most or all of the telomere. This loss of telomere function would explain the high frequency of telomere associations in tissues (61) and cell lines (62) associated with AT, because activation of NBS1 by ATM is required for MRN function (5–7). Similarly, NBS cells have also been reported to have an increased frequency of telomeric fusions (13), consistent with the increased frequency of dicentric chromosomes observed in the cells expressing NBS1<sup>S278A/S343A</sup>.

The sudden loss of a telomere could result from several different mechanisms, including (1) an increase in the rate of DSBs or decreased repair of DSBs in subtelomeric or telomeric regions, (2) an increased rate of homologous recombination in subtelomeric or telomeric regions, or (3) a failure to form a cap on the end of the chromosome although telomeric repeat sequences are present. An increase in the rate of DSBs and an increased rate of homologous recombination could both occur due to problems encountered during DNA replication or a failure to resolve stalled replication forks. In Xenopus, the MRN complex prevents the accumulation of DSBs occurring during DNA replication (63). Similarly, in mammalian cells, MRN foci appear during S phase and are associated with proliferating cell nuclear antigen, a protein involved in DNA replication (64). MRN foci also appear following the inhibition of replication fork progression by UV light in xeroderma pigmentosum variant cells that are defective in bypass of UV-induced DNA damage (65). A decrease in the efficiency of DSB repair in NBS cells would be consistent with the results in chicken DT40 cells, which demonstrate a role for the MRN complex in homologous recombination (13, 14). The involvement of NBS in DNA repair is also demonstrated by the association of NBS1 with BRCAl and phosphorylated H2AX, both of which have been demonstrated to be involved in homologous recombination (21–23). A deficiency in DNA repair would explain the high...
frequency of spontaneous translocations in NBS and the increased cell killing and chromosome damage observed in NBS cells following exposure to ionizing radiation (10). Consistent with this possibility, a recent study has found that increased telomere shortening in AT cells occurs as a result of increased sensitivity to oxidative damage (66). However, in both NBS cells (17, 18) and AT cells (15, 16), most DSBs are repaired; therefore, the exact type of repair defect remains to be determined. Regardless of how they occur, chromosome breaks within the subtelomeric region could result in direct inactivation of the HSV-1k gene, while degradation from breaks that occur within the telomeric repeat sequences could result in inactivation of the HSV-1k gene following degradation if the remaining telomeric repeat sequences are too short to form a functional telomere.

The complete loss of telomeres that we have observed could also be secondary events that result from the inability of cells expressing NBS1\(^{S278A/S343A}\) to properly form a cap that protects the end of the chromosome. In this scenario, complete loss of the telomere and loss of the HSV-1k gene would only occur after breakage of dicentric chromosomes that occur despite the presence of large amounts of telomeric repeat sequences on the end of the chromosome. Fusion without loss of most of the telomeric repeat sequences could occur as a result of the loss of the single-stranded tail on the end of the chromosome, as has been proposed previously with deficiencies in DNA-PKcs (32) and TRF2 (30). Similarly, failure to form T-loops that are proposed to protect chromosome ends through the insertion of the single-stranded tail back into the telomeric repeats (67) could also result in loss of capping function. Consistent with a role for NBS1 in capping function, the MRN complex binds to TRF2 (43) that is thought to be involved in formation of T-loops (45). In this regard, the modification of NBS1 by ATM may be important for telomere replication, because while MRE11 and RAD50 are associated with telomeres throughout the cell cycle, NBS1 is associated with the telomere only during S phase (43). This observation has led to the proposal that the MRE11 complex is involved in either establishment of the single-stranded tail or T-loop formation (43, 68). In fact, we have obtained results similar to those presented here using a dominant-negative WRN gene (69), which has also been demonstrated to bind to TRF2 (70). Consistent with this observation, studies in yeast have found similar shortening of single-stranded telomeric tails in yeast with mutations in either MRE11 or SGS1, the homologue of WRN.\(^1\)Regardless of the mechanism involved, telomere loss due to a deficiency in NBS1 could have severe consequences for chromosome stability, because telomere loss can lead to extensive chromosome rearrangement for many cell generations by initiating B/F/B cycles (46, 47, 53). NBS1 is therefore one of a growing list of proteins that functions to prevent chromosome instability through dual roles in both telomere maintenance and cellular response to DNA damage.

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\(^1\)R. Welling, personal communication.

### Materials and Methods

#### Cell Lines and Culture

The EJ-30 cell line (obtained from Dr. William Dewey, University of California, San Francisco) was subcloned from the EJ bladder cell carcinoma cell line, which is also named MGH-U1 (71). Clone B3 was isolated from EJ-30 following transfection with the pNCT-tel plasmid and contains a single copy of the plasmid integrated at the end of the short arm of chromosome 16 (46, 47). B3 and all B3-derived subclones were grown in α-MEM (UCSF Cell Culture Facility) containing 5% FCS (Hyclone Laboratories), 5% donor calf serum with iron (Hyclone, Logan, UT), and 1 mM l-glutamine (UCSF Cell Culture Facility).

Clone BNmt-On was derived from clone B3 following stable transfection with the NBS1\(^{S278A/S343A}\) gene (5), kindly provided by Dr. Eva Lee (University of Texas, San Antonio). Inducible expression of the NBS1\(^{S278A/S343A}\) gene in BNmt-On is achieved with the RetroTet ART system (kindly provided by Dr. Helen Blau, Stanford University), which uses a transcriptional activator that is active in the presence of tetracycline or doxycycline and a repressor that is inactive in the presence of tetracycline or doxycycline (54). To obtain clone BNmt-On, clone B3 was infected with both the RTRg(−)cd8 retrovirus containing a tetracycline-responsive repressor and the RTAb(+) retrovirus containing a tetracycline-responsive activator. Identification of clones containing the RTRg(−)cd8 retrovirus was accomplished by the presence of CD8 using a CD8-specific antibody. Following infection, 200 cells were replated in 100-mm tissue culture dishes and the cells were incubated for 2 weeks at 37°C. CD8-positive colonies were then detected by first washing the cells at 4°C with PBS (UCSF Cell Culture Facility) containing 0.25% BSA (Sigma Chemical Co., St. Louis, MO) followed by incubation at 4°C for 15 min in 1 ml of PBS containing 0.25% BSA and a 1:100 dilution of CD8-specific antibody (PharMingen, San Diego, CA). The cells were then washed at 4°C with PBS containing 0.25% BSA and screened for CD8 expression by analysis with a fluorescent microscope using a lens immersed directly within the growth medium. Clones expressing CD8 were then isolated, and one of these clones was infected with the RTAb(+) retrovirus. Forty clones selected at random from the RTAb(+)-infected cells were then tested for expression of the activator in RTAb(+) by individually infecting them with the HRSpuro-gfp retrovirus that contains the enhanced green fluorescent protein (EGFP; Clontech Laboratories, Inc., Palo Alto, CA) under the control of the tetracycline-responsive promoter. Following selection with 2 μg/ml puromycin, the stably infected clones were then plated for expression of the activator in RTAb(+) by individually infecting them with the HRSpuro-gfp retrovirus that contains the enhanced green fluorescent protein (EGFP; Clontech Laboratories, Inc., Palo Alto, CA) under the control of the tetracycline-responsive promoter. Following selection with 2 μg/ml puromycin, the stably infected clones were then screened for EGFP expression in the presence of doxycycline using a B-2E/C FITC filter on a Nikon E600 Eclipse fluorescent microscope (Burlingame, CA). Clone B3rr that showed high levels of EGFP in the presence of doxycycline but no EGFP in the absence of doxycycline was then infected with the HRSpuro retrovirus containing either NBS1\(^{S278A/S343A}\) gene or wild-type NBS1 on a tetracycline-responsive promoter. Puromycin-resistant clones were then analyzed for inducible expression of the NBS1\(^{S278A/S343A}\) or wild-type genes using RT-PCR.

Clone BNmt-Off was also derived from clone B3 but uses the Tet-Off system (50) to regulate the NBS1\(^{S278A/S343A}\) gene. The Tet-Off system uses an activator that is inactive in the...
presence of tetracycline or doxycycline. Thus, unlike BNmt-Off, the NBSI S278A/S343A gene in BNmt-Off is expressed in the absence of doxycycline. To obtain clone BNmt-Off, clone B3 was first transfected with pUHD15-1(hph) that contains the tetracycline-responsive activator, and transfected clones were selected with 200 μg/ml hygromycin (Sigma). pUHD15-1(hph) consists of the pUHD15-1 plasmid (kindly provided by Dr. Hermann Bujard, Universität Heidelberg) into which the hygromycin-resistant gene with a SV40 early gene promoter has been inserted. Cells containing the stably integrated activator were then identified by transfection with the pUHD10-3(puro) plasmid containing EGFP gene under the control of the tetracycline-responsive promoter. Clones containing the stably integrated EGFP gene were then selected in 2 μg/ml puromycin. pUHD10-3(puro) was constructed from pUHD10-3 (kindly provided by Dr. Hermann Bujard) by insertion of the puromycin-resistant gene with a HSV-tk gene promoter. The presence of the activator was then determined by the presence of EGFP in absence of doxycycline as determined with a fluorescent microscope. Clones containing the activator were then transfected with the pUHD10-3(puro) plasmid containing the NBSI S278A/S343A gene under the control of the tetracycline-responsive promoter. Puromycin-resistant clones were then analyzed for inducible expression of the NBSI S278A/S343A gene using RT-PCR.

Viral Preparation and Infection
Retroviruses were generated by transient transfection of amphotropic 293 cells (Clontech, Carlsbad, CA) with the retrovirus vectors using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions. Infection of cells with retroviruses was accomplished by incubation of the cells with medium harvested from the amphoteric 293 cells and filtered with a 0.45-μm Acrodisc filter (Gelman Science, Inc., Ann Arbor, MI). Prior to infection, 8 μg/ml of polybrene (Sigma) for 2 min, washed with Tris-buffered saline, and treated with 3.7% formaldehyde in Tris-buffered saline (Sigma). Genomic DNA was isolated from cell cultures according to the manufacturer’s instructions using the StrataPrep Total RNA Miniprep Kit (Stratagene, La Jolla, CA). RT-PCR was then performed using a SuperScript One-Step RT-PCR Kit with Platinum Taq (Invitrogen) according to the manufacturer’s instructions. The RT-PCR conditions involved one cycle at 45°C for 30 min and then at 94°C for 2 min. This was followed by 35 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min, with the final cycle at 72°C for 10 min. The primer sequences specific for the NBSI S278A/S343A and wild-type NBSI transgenes were NB-1 (5'-TGATACAGGAATATTAAACAGC-3') and NB-2 (5'-TCAACTTACAGCTTTTGTC-3') that generate a 230-bp fragment and NB-3 (5'-GGTCGACC-CAGATTGTGCTAGA-3') and NB-4 (5'-GTTTTTGGTTTCTACCGACCGTTGG-3') that generate a 2.2-kb fragment. As a control, RT-PCR was performed simultaneously for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH) using the primers 5'-CATGTTGGCCCATGAGTTCCACAC-3' and 5'-TGAAGGTCCGGAGTCAACG-GATTGGT-3'.

Chromosome and Telomere Analysis
Chromosome analysis was performed following the addition of 0.1 μg/ml colcemid to the medium for 1.5–2 h at 37°C. The cells were then trypsinized, pelleted at 500 × g for 5 min, the supernatant removed, 2 ml of 0.56% KCl added, and incubated for 30 min at 37°C. The cells were then spread on a slide and dried at room temperature prior to analysis.

Telomere analysis was performed using the Telomere PNA FISH Kit/Cy3 (DAKO, Glostrup, Denmark) as described by the manufacturer. Briefly, the metaphase preparations were treated with 3.7% formaldehyde in Tris-buffered saline (Sigma) for 2 min, washed with Tris-buffered saline, and incubated with pretreatment solution for 10 min. The slides were then treated with a cold ethanol series consisting of 70%, 86%, and 95% ethanol. After the slides were dried, 10 μl of telomere PNA probe labeled with Cy3 were added, and a coverslip was placed on top. The slides were then denaturated in a preheated incubator at 80°C and placed in the dark for 30 min at room temperature. The slides were then rinsed with wash solution at 65°C, treated again with the cold ethanol series, and dried. The slides were then prepared for fluorescent microscopy by addition of 50 μl of mounting solution and addition of a coverslip. A minimum of 50 metaphase spreads were scored for each time point, and the values represent an average of two separate experiments.

Fluctuation Analysis and Mutant Selection
For analysis of the rate of loss of the HSV-tk gene, the cells were plated at 100 cells/well in a 24-well culture dish in medium with or without 1 μM doxycycline. The cultures were then expanded to more than 10⁶ cells, after which time 10⁷ were plated into medium containing 50 μM ganciclovir (a kind gift from Hoffmann-La Roche, Inc., Nutley, NJ) and 400 μg/ml G418 (Life Technologies, Inc., Grand Island, NY) for identification of cells that had lost the HSV-tk gene and used for analysis.
Telomerase Activity
Relative telomerase activity was determined using the TelomAGG Telomerase PCR ELISA assay (Roche, Indianapolis, IN) as described by the manufacturer using measurements taken with a spectrophotometer at a wavelength of 450 nm.

Cell Survival and DNA Synthesis
The effects of ionizing radiation on cell survival was determined by plating 200 cells in 100-mm tissue culture dish with three plates for each dose. After exposure of cells to γ-rays (87 cGy/min), cells were cultured for 10 days prior to staining and determining percentage of survival relative to unirradiated controls. Experiments were repeated four to six times.

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Telomere Instability in a Human Tumor Cell Line Expressing NBS1 With Mutations at Sites Phosphorylated by ATM

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