

# Genetic Mapping Using Microcell-Mediated Chromosome Transfer Suggests a Locus for Nijmegen Breakage Syndrome at Chromosome 8q21-24

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## Summary

Nijmegen breakage syndrome (NBS) is an autosomal recessive disorder characterized by microcephaly, short stature, immunodeficiency, and a high incidence of cancer. Cultured cells from NBS show chromosome instability, an increased sensitivity to radiation-induced cell killing, and an abnormal cell-cycle regulation after irradiation. Hitherto, patients with NBS have been divided into the two complementation groups V1 and V2, on the basis of restoration of radioresistant DNA synthesis, suggesting that each group arises from a different gene. However, the presence of genetic heterogeneity in NBS has been considered to be controversial. To localize the NBS gene, we have performed functional complementation assays using somatic cell fusion between NBS-V1 and NBS-V2 cells, on the basis of hyper-radiosensitivity, and then have performed a genomewide search for the NBS locus, using microcell-mediated chromosome transfer followed by complementation assays based on radiosensitivity. We found that radiation resistance was not restored in the fused NBS-V1 and NBS-V2 cells and that only human chromosome 8 complements the sensitivity to ionizing radiation, in NBS cell lines. In complementation assays performed after the transfer of a reduced chromosome, merely the long arm of chromosome 8 was sufficient for restoring the defect. Our results strongly suggest that NBS is a homogeneous disorder and that the gene for NBS is located at 8q21-24.

## Introduction

Nijmegen breakage syndrome (NBS) (MIM 251260; also known as an “ataxia-telangiectasia (AT) variant”) is a fourth-chromosome breakage syndrome, which was discovered after Fanconi anemia, Bloom syndrome, and AT. NBS is an autosomal recessive disorder, and at least 42 patients have been reported since the recognition in 1981 of the first 2 patients (Weemaes et al. 1981). Patients with NBS have a growth retardation of pre- or postnatal onset, severe microcephaly after the first months of life, a typical face with a receding forehead, a prominent midface with a long nose and a receding mandible, and profound immunodeficiency involving both the cellular and the humoral systems. Respiratory-tract infections occur in almost all patients with NBS, and urinary or gastrointestinal infections repeatedly occur in 15% of patients. Patients also have a very high risk of cancer. So far, 12 of 42 patients have developed a lymphoma at a young age (see the review by van der Burg et al. [1996]). Mitogen-stimulated T cells from NBS patients show chromosome instability, such as spontaneous chromosomal breaks, gaps, or rearrangements. Most of the rearrangements preferentially occur in chromosomes 7 and 14, which are the same breakpoints as those found in AT. Cultured cells from NBS also show an increased sensitivity to radiation-induced cell killing and an abnormal cell-cycle regulation after irradiation, such as radioresistant DNA synthesis (RDS). These cell-biological findings very much resemble those in AT, suggesting that the same pathway (or part thereof) is impaired in both syndromes. However, the clinical features are quite different, since patients with NBS do not show elevation of  $\alpha$ -fetoprotein, cerebellar ataxia, or telangiectasia but do have microcephaly and growth retardation.

Patients with NBS have been divided into two complementation groups, V1 and V2, on the basis of restoration of RDS (Jaspers et al. 1988), whereas AT patients have been represented by four other, separate groups—A, C, D, and E—which were determined by the same

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kind of complementation study as that used for NBS (Jaspers and Bootsma 1982; Murnane and Painter 1982). The AT locus was mapped to 11q22-23 (Gatti et al. 1988), whereas the recently cloned AT-mutated (ATM) gene was found to be mutated in all AT patients (Savitsky et al. 1995; McConville et al. 1996), which implies that, at least in AT, complementation studies based on the restoration of RDS obviously gave conflicting results. Although NBS has been categorized historically as an AT variant, haplotype studies of NBS families (Green et al. 1995; Stumm et al. 1995) and complementation studies based on the hyper-radiosensitivity of NBS cells (Komatsu et al. 1996) both have demonstrated that the underlying gene(s) for NBS is distinct from the ATM gene. Thus, the prevailing of two complementation groups in NBS has been considered to be controversial, and the chromosomal location of the NBS gene remains to be clarified.

The microcell-mediated chromosome-transfer technique is a well-known approach for the identification of a disease-bearing chromosome (Komatsu et al. 1990; Jongmans et al. 1995; Whitney et al. 1995). To localize the NBS gene, we reassayed the complementation groups in NBS by functional assays based on hyper-radiosensitivity, using somatic cell fusion between NBS-V1 and NBS-V2 cells, and performed a genomewide search for the NBS locus, using microcell-mediated chromosome transfer followed by complementation of the restoration of radiosensitivity (Komatsu et al. 1993). Here, we report that NBS is a homogeneous disorder and that the NBS gene maps to chromosome 8q. Identifying the NBS locus on chromosome 8q will facilitate the positional cloning of the NBS gene.

## Material and Methods

### *Cell Lines and Culture Conditions*

An immortal cell line, GM7166VA7, from skin fibroblasts of a patient belonging to NBS-V2 (Conley et al. 1986) had been established in our laboratory (Komatsu et al. 1996). An immortal cell line, 1022QVA8N, was established, by use of SV40 transformation, from a skin fibroblast of an NBS-V1 patient at Nijmegen University. The human/mouse hybrid cell lines GM13139, GM11686, GM11713, GM11687, GM11714, GM11688, GM13259, GM11689, GM11715, GM13260, GM10479B, and GM13258 were obtained from the NIGMS Human Genetic Mutant Cell Repository. The human/mouse hybrids A9(neo6)-3, A9(neo7)-2, A9(neo8)-1, A9(neo11)-1, A9(neo16), A9(neo17)-11, A9(neo18)-5, A9(neo19)-4, and A9(neo21) were constructed previously. All cell cultures were maintained at 37°C and 5% CO<sub>2</sub>, in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone) and 20 mg gentamycin/ml.

### *Somatic Cell Fusion*

Functional complementation was assayed by somatic cell fusion either between the 1022QVA8N(neo) and GM7166VA7(hygro) cells or between the 1022QVA8N(bsr) and GM7166VA7(neo) cells, as well as between the 1022QVA8N(neo) cells and the AT5BIVA(bsr) cells belonging to the classic AT-D complementation group, as described in a previous study (Komatsu et al. 1996). The suffix "neo," "hygro," or "bsr" indicates the cells tagged with pSV2neo, pSV2hygro, or pSV2bsr, respectively. Cell fusion was performed by treatment with 50% polyethylene glycol 1000 (Baker) for 1 min and then by incubation in the presence of selection medium containing 800 mg G418/ml and either 300 mg hygromycin/ml or 5 mg blasticidin/ml (Gibco). The resulting hybrid clones were isolated by use of a metal cloning cylinder.

### *Microcell-Mediated Chromosome Transfer*

Microcell-mediated chromosome transfer was performed by use of a method described in a previous study (Komatsu et al. 1990). In brief, donor A9 cells containing a single human chromosome were plated onto 25-cm<sup>2</sup> flasks and were allowed to recover for 1 d or for 2 d. Then, Colcemid (Sigma) was added to the medium at a concentration of 0.05 mg/ml. After 48 h of incubation, enucleation was performed by centrifugation at 8,000 cpm for 1 h, in the presence of 10 mg cytocharasin B/ml (Sigma). The microcell pellet was recovered, resuspended in serum-free medium, and filtered through a series of 8-mm, 5-mm, and 3-mm polycarbonate-membrane filters (Nucleopore). The purified microcells were allowed to attach to the surface of a monolayer of recipient GM7166VA7 cells, in the presence of a medium containing 100 mg phytohemagglutinin/ml (Difco). After 15 min, the cells were fused by treatment with 50% polyethylene glycol 1000 (Baker) for 1 min and were allowed to grow in DMEM with 10% fetal bovine serum. The next day, the cells were trypsinized and plated into three 100-mm plates containing 800 mg G418/ml. After 3 wk, the resulting microcell-hybrid clones were isolated.

### *Cell-Survival Assay*

Cells were plated onto 100-mm dishes and were allowed to grow to an 80% confluence. The cells were trypsinized and irradiated with 4 Gy of <sup>60</sup>Co  $\gamma$ -rays at a dose rate of 55 cGy/min. Immediately after irradiation, the cells were plated onto 100-mm dishes at a cell density that would allow 30-40 cells to survive and were incubated for 12 d. The dishes were fixed with ethanol and were stained with 4% Gimsa, and the number of colonies was counted. The data represent an average value from two experiments.

### Irradiation Microcell-Mediated Chromosome Transfer

The generation and purification of microcells from the A9(neo8)-1 cells were performed as described above. The purified microcells were irradiated with a dose of 5 Gy of  $^{60}\text{Co}$   $\gamma$ -ray at a dose rate of 55 cGy/min and immediately were fused to recipient murine A9 cells. After selection with G418, the resultant microcell hybrids were isolated and were expanded.

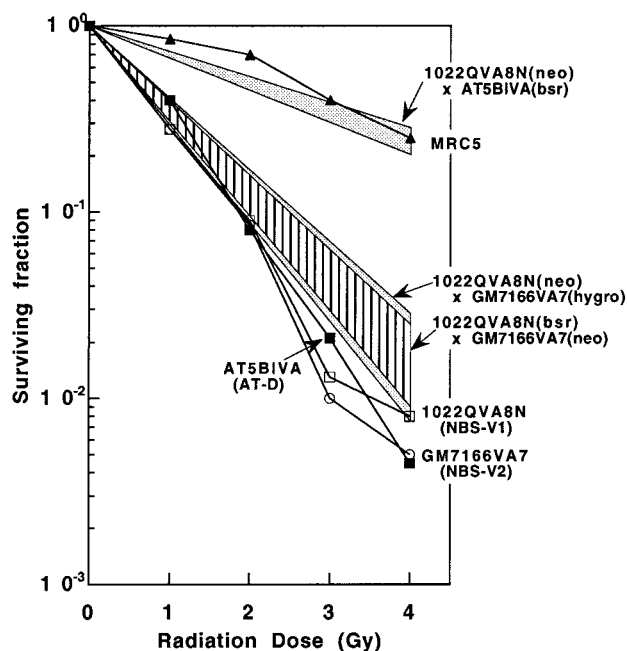
### Microsatellite PCR

PCR primers were synthesized in accordance with the sequence-tagged site (STS) sequence data or were obtained commercially from Research Genetics. Genomic DNAs from GM7166VA7 microcell hybrids were mixed with 10 pmol of each primer, 1.0 pmol of one primer end-labeled with [ $\gamma$ - $^{32}\text{P}$ ] ATP (Amersham), 0.2 mM of each dNTP, and 0.25 units of *Taq* DNA polymerase (Takara), in a total volume of 10  $\mu\text{l}$ . The samples were incubated at 94°C, 58°C, and 72°C, for 30 s at each temperature, in an Astec PC-800 for a total of 35 cycles. PCR products were separated on 6% polyacrylamide/urea gels containing 30% formamide. The gels were fixed and were analyzed by use of the BAS-1500 Mac bio-imaging analyzer (Fujifilm). DNAs from irradiation-reduced microcell hybrids were PCR amplified as described above, without primer end labeling. PCR products were electrophoresed on 2% agarose gels, were stained with ethidium bromide, and were photographed.

## Results

### Genetic Homogeneity in NBS

An immortalized cell line, 1022QVA8N, from a skin fibroblast of an NBS-V1 patient was established by use of SV40 transformation. An immortalized fibroblast cell line, GM7166VA7, from a patient with NBS-V2 was described in a previous study (Komatsu et al. 1996). The AT5BIVA cell line from an AT-D patient also was used as a control for somatic cell fusion. Complementation assays using somatic cell fusion between NBS-V1 and NBS-V2 cells were performed on the basis of radiation sensitivity to cell killing. Both neo- and hygro-tagged clones of 1022QVA8N and GM7166VA7 cells were highly sensitive to radiation, as compared with normal MRC5 cells. After cell fusion either between 1022QVA8N(neo) and GM7166VA7(hygro) cells or between 1022QVA8N(hygro) and GM7166VA7(neo) cells, a total of eight hybrid clones were obtained. All clones lacked any significant restoration of radiation sensitivities, although a small increase in the surviving fraction was observed (fig. 1). In contrast, when 1022QVA8N(neo) cells were fused with AT5BIVA(bsr) cells, all hybrid clones exhibited a full restoration of radiation resistance. These results suggested that the

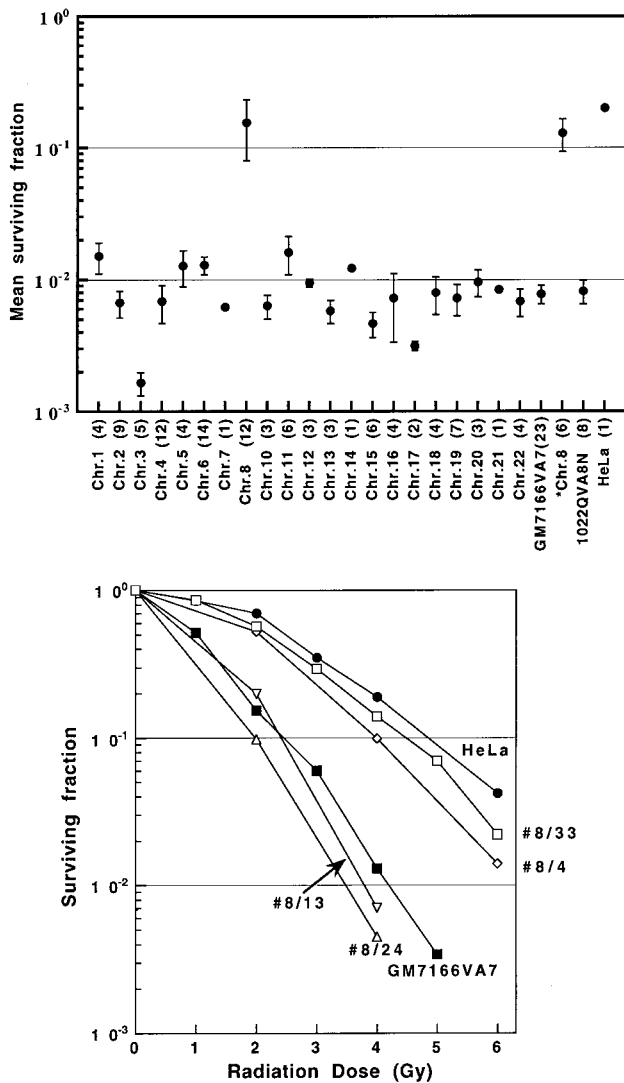


**Figure 1** Results of functional complementation assays between NBS-V1 and NBS-V2 cells. The survival curves for  $\gamma$ -ray-irradiated 1022QVA8N (NBS-V1), GM7166VA7 (NBS-V2), AT5BIVA (AT-D), and normal MRC5 cells are shown. Surviving fractions for the 4-Gy  $\gamma$ -ray-irradiated hybrid cells 1022QVA8N(neo)  $\times$  GM7166VA7(hygro) and 1022QVA8N(bsr)  $\times$  GM7166VA7(neo) are shown within a shaded area or within a hatched area, respectively. Surviving fractions for the 4-Gy  $\gamma$ -ray-irradiated hybrid cells 1022QVA8N(neo)  $\times$  AT5BIVA(bsr) also are shown within a shaded area, as indicated. The hybrid clones of 1022QVA8N(neo)  $\times$  GM7166VA7(hygro) and of 1022QVA8N(bsr)  $\times$  GM7166VA7(neo) lacked the restoration of radiation sensitivity, whereas the hybrid clones of 1022QVA8N  $\times$  AT5BIVA showed a full restoration of radiosensitivity.

underlying gene for NBS-V1 is the same as that for NBS-V2.

### Complementation by Chromosome 8

To identify the disease-bearing chromosome, microcell-mediated chromosome transfer was performed. An immortalized fibroblast cell line, GM7166VA7 (from an NBS-V2 patient), was used as a recipient for microcell fusion. A library of murine A9 hybrid cell lines containing a single human chromosome tagged with a neo gene was used as a chromosome donor. Microcells were produced from the murine A9 hybrid cells and were fused to the GM7166VA7 cells. After G418 selection, the resulting microcell hybrids were isolated and were used for complementation assays based on radiation sensitivity at 4 Gy. Since NBS shows autosomal recessive inheritance, all autosomal chromosomes, except for chromosome 9, were tested. Chromosome 9 donor cells tagged with neo were not available. In each experiment, 1–12 clones of the microcell hybrids were obtained. Complementation studies demonstrated that none of the



**Figure 2** *Top*, Results of complementation assays of GM7166VA7 microcell hybrids with a series of transferred human chromosomes. The plots indicate the mean surviving fraction of microcell hybrids after 4 Gy of  $\gamma$ -ray irradiation. Each vertical bar represents the standard error of the mean. The numbers in parentheses indicate the number of hybrids that were analyzed. HeLa cells were used as a normal control. “\*Chr.8” represents the surviving fraction of 1022QVA8N hybrid clones with a transferred chromosome 8. Only the introduction of human chromosome 8 corrected the hyper-radiosensitivity of the GM7166VA7 cells, as well as that of the 1022QVA8N cells. *Bottom*, Survival curves for  $\gamma$ -irradiated GM7166VA7 cells, control HeLa cells, and microcell hybrids transferred with a chromosome 8. Data for two clones (#8/4 and #8/33) of functionally complemented hybrids and for two clones (#8/13 and #8/24) of noncomplemented hybrids are shown.

microcell hybrids containing human chromosomes 1-7 or 10-22 showed restoration of radiation resistance. In contrast, 9 of 12 microcell-hybrid clones with a transferred human chromosome 8 were rescued, since radiation resistance was normalized (fig. 2, *top* and *bottom*). To confirm this, we performed a second transfer of a

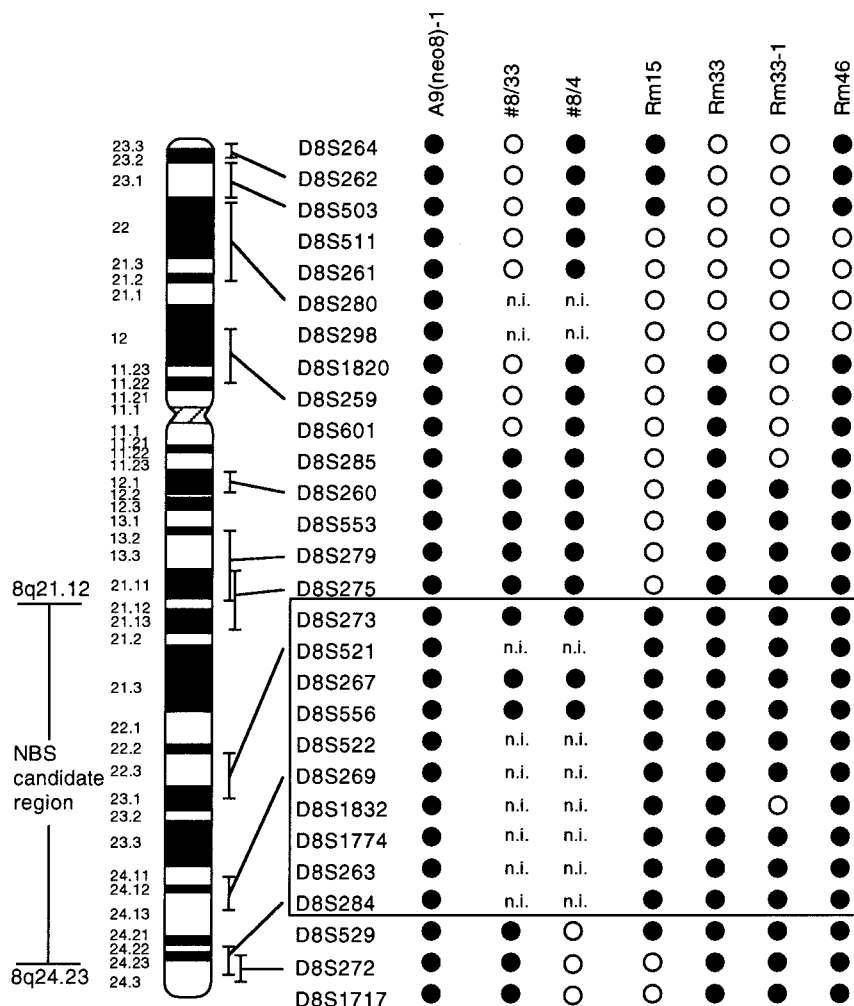
chromosome 8 and generated an additional six microcell hybrids. All six hybrids showed normal levels of radiation sensitivity (data not shown). Chromosome painting of these complemented hybrids was performed by use of mouse genomic DNA as a probe. No signals were detected, excluding the possibility of the presence of mouse DNA and, thus, of a cotransfer of mouse chromosomes. In addition, when a chromosome 8 was transferred into 1022QVA8N cells (from an NBS-V1 patient), six clones of microcell-hybrid cells were obtained. Four of the six microcell-hybrid clones showed the restoration of radiation sensitivity (fig. 2, *top*). These data clearly demonstrate that only human chromosome 8 complements the sensitivity to ionizing radiation in NBS cell lines.

*Deletion Mapping of Chromosome 8*

During microcell-mediated chromosome transfer, donor chromosomes frequently delete or rearrange (Leach et al. 1989). Therefore, we performed deletion mapping for 13 clones of complemented chromosome 8 hybrids and for 3 clones of noncomplemented hybrids. As the donor chromosome and the recipient GM7166VA7 cells were both of human origin, we utilized CA-repeat polymorphisms that were present on chromosome 8. Of 28 STS loci analyzed, 19 were informative. Eleven (85%) of 13 clones of complemented hybrids were positive for all 19 STS markers, indicating the successful transfer of an entire human chromosome 8, whereas the remaining 2 clones (15%) had lost some markers. Complemented hybrid clone #8/33 was negative for all markers on chromosome 8p, and clone #8/4 was deleted of markers in the distal part of the long arm (fig. 3). Therefore, the underlying gene for NBS has to be excluded from these regions. In contrast to the complemented hybrids, all three clones of noncomplemented hybrids showed large deletions. Noncomplemented hybrid clones #8/21 and #8/24 were negative for all 19 markers, whereas clone #8/13 showed deletions in the distal long arm that were comparable to the pattern shown in complemented hybrid clone #8/4. Analysis of the distal breakpoints of restored clone #8/4 and of nonrestored clone #8/13 may be useful for a more precise mapping of the NBS locus. However, as yet, we have not been able to find any informative markers in the distal long arm of chromosome 8. The results obtained thus far suggest that the NBS gene must be located in the centromeric region or in the long arm of chromosome 8.

*Complementation by Chromosome 8q*

To narrow the NBS candidate region, we generated a new series of murine A9 hybrid cell lines, which contained a deleted human chromosome 8. The irradiation microcell-mediated chromosome transfer has been proven to be useful for the generation of a reduced chro-



**Figure 3** Ideogram of human chromosome 8 and deletion map for the NBS-V2 locus. Complemented hybrid clones (#8/4 and #8/33) and irradiation-reduced A9 microcell hybrids (Rm15, Rm33, Rm33-1, and Rm46) were analyzed by microsatellite PCR. Blackened and unblackened circles indicate the presence or absence, respectively, of STS markers. n.i. = not informative. Hybrid clone #8/33 had deletions of 8p markers, and clone #8/4 was deleted at the distal long arm of chromosome 8. Rm15 cells had deletions around the centromeric region, and Rm33, Rm33-1, and Rm46 cells had deletions in the short arm of chromosome 8. The common conserved region is surrounded by a box, and the NBS candidate region is indicated.

mosome (Dowdy et al. 1990). Microcells from A9(neo8)-1 cells were irradiated with 5 Gy of  $\gamma$ -rays and were fused back to murine A9 cells. After G418 selection, 50 colonies were isolated and were analyzed for their human chromosome content. These hybrids initially were screened by use of 17 selected STS primers from chromosome 8. Of the 50 clones, 20 appeared positive for all 17 markers, suggesting no gross deletion. The other 30 hybrid clones showed several patterns of deletions. Four of these hybrid clones (Rm15, Rm33, Rm33-1, and Rm46) showed a large contiguous deletion of at least 4 STS markers. By use of all 28 STS markers, a more precise deletion mapping was performed (fig. 3). Clone Rm15 was negative for the markers surrounding the centromeric region of chromosome 8, whereas the Rm33, Rm33-1, and Rm46 cells were negative for mark-

ers in the short arm. Hybrid Rm33-1 also had a microdeletion around locus D8S1832. FISH analysis using human genomic DNA as a probe indicated that these reduced chromosomes were not translocated to a mouse chromosome. We then introduced the reduced chromosomes into GM7166VA7 cells via microcell fusion, and we analyzed the radiosensitivity of the resultant microcell hybrids. Rm15 cells generated six microcell-hybrid clones, which showed restoration of radiation resistance. Three clones of the four microcell hybrids obtained from Rm33, two clones of the two hybrids from Rm33-1, and four clones of the four hybrids from Rm46 all showed restoration of radiation resistance (results not shown). These data indicate that the NBS gene is located neither in the short arm nor in the centromeric region of chromosome 8. Considering the results ob-

tained from the hybrids of clones #8/33 and #8/4, we conclude that the NBS gene resides in the distal long arm of chromosome 8—most probably in the region 8q21.12-24.23.

## Discussion

We examined the genetic heterogeneity of NBS-V1 and NBS-V2, by somatic cell fusion between two cell lines. Previously, NBS-V1 and NBS-V2 were defined as belonging to different complementation groups, on the basis of the restoration of RDS in heterodikaryons generated by somatic cell fusion (Jasper et al. 1988). We have reported the phenotypic separation between radiation sensitivity and RDS, in synkaryons generated from somatic cell fusions of immortalized AT cell lines, for which the radiation-sensitivity phenotype was recessive but for which RDS appeared dominant in the hybrids of AT cells  $\times$  normal cells (Komatsu et al. 1989). Similarly, dominant expression of RDS was observed in AT-like hamster cells, designated "V-E5," when a mouse chromosome complementing the radiation sensitivity of the V-E5 cells was transferred into these cells (Zdzienicka et al. 1994). Both experiments suggest that the abnormal cell-cycle regulation of AT may be modified by cell fusion but that the radiation sensitivity is maintained as an intrinsic phenotype, since AT is a recessive disease. In the work reported here, we used radiation sensitivity, instead of RDS, as a biological marker for a complementation assay. The results strongly suggested that only a single gene responsible for NBS appears to be present. As expected from these results, the radiosensitivity of the newly established NBS-V1 fibroblast cell line was complemented by the transfer of a chromosome 8. These results support the theory that the underlying gene of NBS-V2 is the same as that of NBS-V1.

Microcell-mediated chromosome transfer is becoming more useful for identification of a disease-bearing chromosome if the cells have a selectable phenotype (Komatsu et al. 1990; Jongmans et al. 1995; Whitney et al. 1995). Functional cloning using cDNA transfection also has been applied to identification of the disease gene. More than 20 human cDNAs that complement radiosensitivity in AT cells were identified by cDNA transfection (Meyn et al. 1993; Jung et al. 1995). However, in transfected cells, this method may, in some cases, identify irrelevant cDNAs as high-copy suppressors of the radiosensitive phenotype, because of overexpression of the cDNA. Since microcell-mediated chromosome transfer utilizes a normal expression of a single-copy gene on the introduced chromosome, the possibility of false-positive results seems to be reduced (Whitney et al. 1995). We note, however, that microcell-mediated chromosome transfer also can lead to inaccurate mapping under some circumstances. In one report, the microcell

transfer of human chromosome 5 conferred enhanced levels of resistance to UV irradiation in xeroderma pigmentosum group C (XPC) cells (Kaur and Athwal 1992), although the correct location of the underlying XPC gene is chromosome 3 (Legerski et al. 1994). This raised the possibility that even an increased number of some chromosomes could influence phenotypic correction in the cells, probably owing to gene dosage effects on the transferred chromosome. To avoid such a possibility of false-positive results, we have performed a genomewide search for the NBS locus, using microcell-mediated chromosome transfer followed by complementation assays. Only human chromosome 8 conferred a full restoration of radiosensitivity in NBS-V2 cells, whereas no complementation was observed by the transfer of the other chromosomes, suggesting that the complementing gene on chromosome 8 is indeed the NBS gene. Furthermore, chromosomal analysis of our NBS cell lines supported the notion that the phenotypic correction observed in NBS cells did not result from an increased number of chromosome 8. Both cell lines, GM7166VA7 and 1022QVA8N, already had contained an increase of three copies of chromosome 8, after an immortalization process (data not shown). However, the radiosensitivity of these NBS cell lines was the same as that of the parental primary cultures (Komatsu et al. 1996). This also suggested that the candidate gene on chromosome 8 has been mutated, and a transfer of a normal NBS gene could normalize the radiosensitivity of NBS cells.

Functional complementation using the microcell-mediated chromosome transfer enabled us to localize a gene for NBS to the long arm of chromosome 8. The ATM gene shows homology with the yeast Tel1p, Mec1p, and Rad3 genes and with the *Drosophila* MEI-41 gene, all of which belong to the PI3 kinase family (Keith and Schreiber 1995; Lavin et al. 1995; Zakian 1995). Since the biological findings in NBS cells resemble those in AT cells, the underlying gene for NBS might be the PI3 kinase-family gene. Recently, a functional counterpart, FRP1, of the Tel1p, Mec1p, and Rad3 genes was isolated (Cimprich et al. 1996). Sequence similarity suggested that the gene may be involved in the detection of and the response of cells to damaged DNA and should be a candidate for NBS. However, the FRP1 gene was mapped to the chromosomal locus 3q22-q24 (Cimprich et al. 1996). Several genes associated with DNA repair have been mapped to chromosome 8. The underlying gene for mouse severe combined immunodeficiency, the DNA-PK<sub>CS</sub> gene, was mapped to 8q11 (Blunt et al. 1995); the WRN gene for Werner syndrome was mapped to 8p11-12 (Yu et al. 1996); and DNA polymerase  $\beta$  was mapped to 8p11-12 (Cannizzaro et al. 1988). These genes are located outside the NBS region and are excluded as candidate genes. At present, we cannot nar-

row down the NBS candidate region any further. Linkage analysis focused on 8q will be of use for a precise mapping of the NBS gene. Then, functional complementation using the introduction of YAC fragments into immortalized NBS cells will facilitate the positional cloning of the NBS gene, which should provide important insight into the mechanisms of DNA repair and carcinogenesis.

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