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Positional Cloning and Functional Analysis of the Gene Responsible for Nijmegen Breakage Syndrome, *NBS1*

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NBS1/Nijmegen breakage syndrome/DNA repair/ MRE11/Ataxia-telangiectasia

Nijmegen breakage syndrome (NBS) is a rare autosomal recessive disorder characterized by microcephaly, combined immunodeficiency, and a high incidence of lymphoid tumor. Cells from NBS patients show chromosomal instability, hypersensitivity to ionizing radiation and abnormal p53-mediated cell cycle regulation. We cloned the underlying gene for NBS, designated *NBS1*, by complementation-assisted positional cloning from the candidate region 8q21. Large genomic sequencing, as well as a search using computer programs, provides a powerful approach for identifying the underlying gene for a disease. The *NBS1* gene encodes a protein of 754 amino acids that has FHA and BRCT domains which often are conserved in cell-cycle checkpoint proteins. The gene has weak homology to the yeast (*Saccharomyces cerevisiae*) Xrs2 protein in the N-terminus region. Like yeast Xrs2, the NBS1 protein forms a complex with hRAD50/hMRE11, and the complex is condensed as foci in the nucleus after irradiation, indicative that this triple-complex is a crucial factor in DNA repair. Functional analysis of the NBS1 protein is in progress and it should provide further clues to understanding the repair mechanism of radiation-induced DNA double-strand breaks.

INTRODUCTION

Repair of DNA double-strand break (DSB) is an important process in maintaining genomes against DNA-damaging agents such as ionizing radiation or DNA cross-linking agents. Over the past decade, many genes related to DSB repair have been identified in mammalian cells. Most belong to X-ray repair cross complementing (XRCC) groups known to be the genes responsible for radiation sensitivity in rodent mutants[1,2]. Although XRCC groups originally were classified based on cell fusion results for hamster radiation-sensitive mutants, some XRCC genes were found to be identical to those that cause hyper-sensitivity of mouse cells to radiation[3]. Of these, XRCC 5, 6, and 7 are components of the DNA-dependent protein kinase (DNA-PK) which acts

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in non-homologous end joining (NHEJ) in DSB repair[4]. A DNA-PK deficiency also causes the mouse *scid* (severe combined immunodeficiency) mutation because of the impaired V(D)J recombination[5], indicative that DSB repair components also are involved in other DNA processing-related cellular functions.

The signal transduction pathway that responds to DNA damage also is a critical factor affecting radiation sensitivity because a radiation-sensitive human disease, ataxia-telangiectasia (AT), shows abnormal signaling after radiation involving cell cycle checkpoint. The ATM (AT-mutated) gene responsible for AT is a protein kinase activated by radiation-induced DNA damage[6]. Many proteins have been identified as targets of ATM kinase, e.g., p53, c-Abl[7], BRCA1[8], and Chk2[9,10]. In particular, phosphorylation of p53 is the regulating pathway of the G1 cell cycle checkpoint or apoptosis for adaptation to genotoxic stress[11]. This is supported by evidence that wortmannin, an inhibitor of PI-3 kinases such as ATM, suppresses the accumulation of p53 after irradiation[12] and that the cellular radiation sensitivity of p53 null cells is altered by the expression of p53 although it depends on mutation position[13].

Only two diseases, AT and Nijmegen breakage syndrome (NBS), are known to be radiation-sensitive hereditary human disorders. Because both have many similar clinical- and cellular-phenotypes, NBS has been classified as a variant of AT. Four complementation groups (A, C, D, and E) have been defined in AT, based on complementation of abrogation of the S-phase check point, the so-called radiation-resistant DNA synthesis (RDS)[14]. Although the four AT complementation groups suggest the existence of identical numbers of genes, only ATM is reported to be solely responsible for all of the AT complementation groups[7]. Therefore NBS also has been speculated to be caused by some *ATM* mutation or alteration of expression. We previously showed that the introduction of a single chromosome 11, which carries a normal *ATM* gene, could not complement the radiation-sensitivity of NBS cells[15], evidence that the gene for NBS is distinct from *ATM*. We therefore attempted to clone and analyze the NBS gene in order to understand the mechanism of repair or signaling after DSB generated by ionizing radiation. This review introduces our study of complementation-assisted positional cloning and the functional analysis of NBS protein in relation to DSB repair.

NIJMEGEN BREAKAGE SYNDROME

Nijmegen breakage syndrome (NBS) is the fourth chromosomal breakage syndrome to be discovered after Fanconi's anemia, Bloom syndrome, and AT[16]. Patients with NBS are characterized as having severe microcephaly after the first months of life; growth retardation of pre- or postnatal onset; a typical face showing a receding forehead, prominent midface with a long nose, and receding mandible; and combined immunodeficiency[17]. They do not have ataxia, cerebral degeneration, telangiectasia, or an accumulation of alpha-fetoprotein, the typical clinical phenotypes of AT. In addition, the cancer risk of about 35% for patients with NBS is markedly higher than the 10–15% of risk for those with AT. The disease appears to be prevalent among eastern and central European populations, in particular among the Polish people[17]. Up to now, 55 patients have been registered at the University Hospital of Nijmegen (Weemaes, *personal com-*

munication). No NBS patient has been reported in the Japanese population. As in AT, cells from NBS patients show chromosomal instability, hypersensitivity to ionizing radiation, and abnormal cell cycle regulation after irradiation; e.g., delayed and lower levels of p53 accumulation[7,18,19], a defect in the G2/M checkpoint[7,19,20], and radiation-resistant DNA synthesis (RDS) known as abrogation of S phase check point[7]. The *NBS1* gene (GenBank #AB013139), which mutated in all NBS patients, has been cloned using two approaches; positional cloning from the candidate region 8q21[21,22] and by searching for the human homologue of the yeast Xrs2 protein[23].

COMPLEMENTATION-ASSISTED POSITIONAL CLONING

We cloned the *NBS1* gene by complementation-assisted positional cloning (CAPC) using immortalized cell line derived from an NBS patient[21]. Figure 1 shows the scheme to search for the gene by CAPC. The method is based on complementation of the patient's cellular phenotypes by introduction of the candidate genome region and genes. CAPC enables us to identify a gene for a rare autosomal recessive disorder, for which conventional positional cloning that uses familial linkage analysis is difficult. We first introduced a single normal human chromosome to the NBS patient cell line by micro-cell fusion and found that complementation of radiation sensitivity occurred only when normal chromosome 8 was transferred to the cells[24]. Then we attempted to narrow down the candidate region using a complementation assay with fragmented chromosome 8 and by haplotype analysis of NBS patient's families. This resulted in the mapping

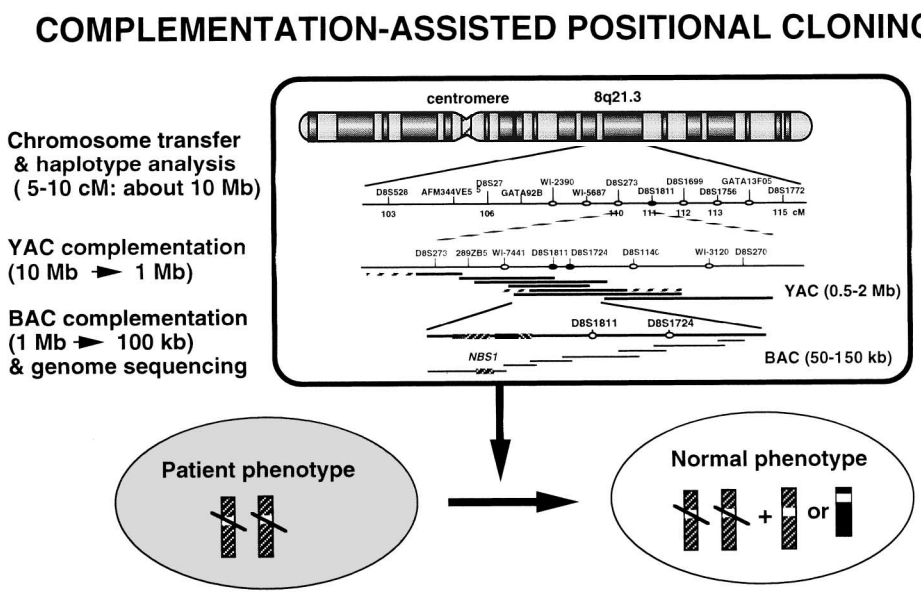

Fig. 1. Scheme of complementation-assisted positional cloning (CAPC). The approach is based on complementation of patient cellular phenotypes by introduction of a chromosome or candidate genomic sequences (YAC, BAC, etc) containing a normal responsible gene. See text for details.

of the NBS gene at a 1.1 cM between STS markers D8S1707 and D8S1818 on chromosome 8q21. Subsequently, we constructed yeast artificial chromosome (YAC) contigs, and the YACs 759G8 and 904D2 from the candidate region were transferred to NBS patient cells. Because only YAC 759G8 restored the radiation resistance of the cells, the candidate region was limited to an 800-kb genomic DNA bearing the STS markers D8S1724 and D8S1811. A bacterial artificial chromosome (BAC) contig which covered the entire 800-kb candidate region was constructed, after which, complementation analysis by the transfection of BAC to NBS cells and large genomic sequencing, were used to search for the candidate gene.

LARGE GENOMIC SEQUENCING AND EXON PREDICTION

Several techniques have been developed to clone cDNAs from large genomic DNA stretches; e.g., exon trapping, cDNA capture with immobilized YACs or BACs[25,26], direct cDNA library screening using labeled YAC DNA as a probe[27], and a combined method using shotgun sequencing and computer-assisted analysis. Using the cDNA library screening technique, we found two genes, *hT41*/*C8orf1* and *DECR*, in this candidate region but they were not mutated in NBS cells. This pre-screening of the gene suggested that a more efficient cloning technique was needed to identify the *NBS1* gene in the critical region. The recent development of refined computer programs, together with the many EST sequences available from public databases, enabled us to search for all the genes and their organization in a large multi-hundred kb genome region. To clone the *NBS1* gene, we attempted to sequence the entire NBS critical genomic region by the shotgun method. Five BAC clones covering 596,533 bp of the 800-kb critical region were sequenced and combined with a BAC sequence from a public database. Totally a 755,832 bp sequence was analyzed by computer programs to identify the possible exons and gene organization. Four genes were identified: *NBS1*, *DECR*, the 27 kDa calbindin gene (*CALB1*), and a novel gene, *hT41*/*C8orf1*. All four were aligned on BAC 255A7 and 157K21 in the 200-kb centromeric region of the sequence[28].

Sequence analysis with the GRAIL II[29] and GENSCAN[30] programs was very effective for predicting or finding the exons and organization of the genes in several 100-kb genome sequences. Forty of a total 43 exons (93%) in the four genes of the candidate region were identified by these two computer programs[28]. Both GRAIL II and GENSCAN found at least one or more exons in each gene having multiple exons, indicative that all genes are detectable by this computer analysis. In addition, many of the EST sequences available from the public database facilitate gene cloning because more than half of the total sequence of the NBS1 cDNA has already been registered as various small independent EST sequences. We conclude that the combination of exon prediction by computer programs such as GRAIL II and an EST search of public databases through the internet is a most efficient approach for cloning a candidate gene from a several 100-kb genomic segment.

THE *NBS1* GENE

The *NBS1* gene contains 16 exons in the more than 48,979 bp of the genomic sequence and is transcribed as two mRNAs of 2.6- and 4.8-kb, which differ in the lengths of their 3'-untranslated region[21,22,28]. This gene encodes 754 amino acids, and the protein shows weak (29%) identity to yeast Xrs2 protein only in the fork head-associated (FHA) domain and BRCA1 C-terminus (BRCT) domain at the N-terminus region (Fig. 2)[21–23].

Six mutations of *NBS1* gene have been reported in NBS patients (Fig. 2). Among them, 657del5 is the most widely spread founder mutation[21,22,31] which causes the largest truncation of the NBS1 protein by a frameshift. The other 5 mutations were also found between codon 233 and codon 385[22], and they cause protein truncation downstream of the FHA/BRCT domains. Therefore, all NBS patients must lose half of the NBS1 protein on the C-terminal side, even though truncated protein is expressed. The amino acid sequence at the C-terminal, more than 70% of the protein, shows no homology with any known protein (including yeast Xrs2 protein), whereas the entire amino acid sequences of the hMRE11 and hRAD50 proteins, components of the NBS1 triple complex, are widely conserved from yeast to mammals.

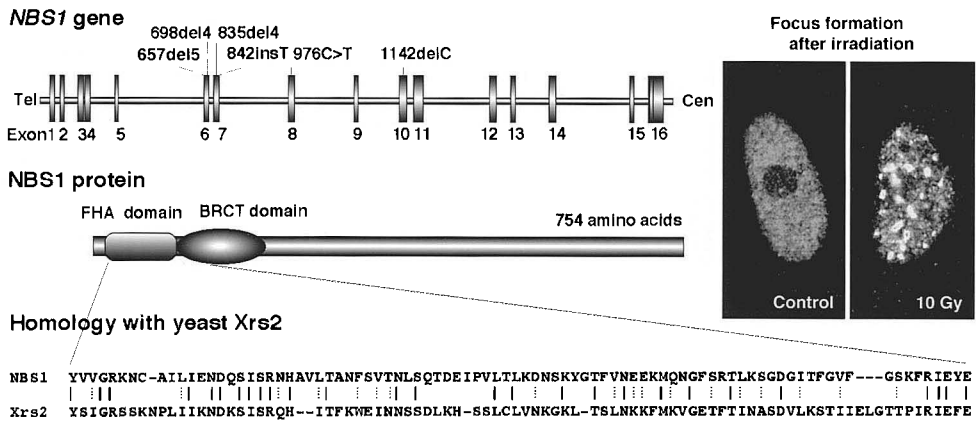

Fig. 2. Structure of the *NBS1* gene and NBS1 protein. NBS1 foci formed in the nucleus after radiation exposure in a normal fibroblast is shown.

POSSIBLE ROLES OF THE NBS1 PROTEIN

Like yeast Xrs2, the NBS1 protein interacts with hMRE11 to form a hRAD50/hMRE11/NBS1 complex[23]. In addition, foci of that protein complex can be seen in the irradiated area of the nucleus[32]. Foci appear within 30 min after radiation exposure and remain in the nuclei even 8 h post-irradiation (Fig. 2)[20]. The number of foci vary with the irradiation dose, suggesting that

they are associated with the site of radiation-induced DSBs[20].

ATM is a regulator both of the cell cycle checkpoints and DSB repair through signal transduction[7]. Since *ATM* mutation causes multiple cellular defects, the gene may act in the early stage of DSB repair signaling[7]. The hRAD50/hMRE11/NBS1 focus formation after irradiation is markedly reduced in AT cells[33], suggesting that NBS1 and ATM share, at least in part, a pathway for DSB repair, possibly the early stage of the DSB repair pathway. It has been reported that NBS1 is essential for the phosphorylation of hMRE11 on DNA damage[34] and that it also potentiates ATP-driven DNA unwinding and endonuclease cleavage by the hRAD50/hMRE11 complex[35]. These findings indicate that NBS1 is a regulatory factor in the DNA processing complex, hRAD50/hMRE11/NBS1, that responds to DNA damage by radiation. In yeast *xrs2* mutants, decreased NHEJ activity[36,37], impaired meiotic recombination[38,39], and abrogation of G2/M checkpoint[40] have been reported. Those phenotypes in *xrs2* yeast seem to be similar to NBS cellular phenotypes. Taking into account the recent findings on NBS1 and reported functions of the yeast Rad50/Mre11/Xrs2 complex[41,42], that complex may act as a key protein in the processing of the DSB end to initiate non-homologous end joining, and perhaps homologous recombination.

In spite of its functional homology to yeast Xrs2, about 70% of the NBS1 protein on the C-terminus side does not show any sequence homology to known proteins including Xrs2[21]. The functions of most domains of the NBS1 protein therefore are still not clear, except for about 200 amino acids of the N-terminus region, known as the FHA and BRCT domains, which may be related to DNA repair, gene regulation, and the cell cycle checkpoint[43,44]. As all the NBS1 mutations in NBS patients occur between codons 220 and 385, they provide truncated proteins downstream of the FHA/BRCT domains. The presence of these domains in all NBS patients suggests that they may be essential at a developmental stage such as embryogenesis.

PERSPECTIVES

Recently, we reported that the expression of the full length NBS1 protein results in the complementation of NBS multiple phenotypes such as radiation sensitivity, the G2 checkpoint, and focus formation after irradiation[20]. These findings indicate that NBS1 is the only functionally responsible protein for NBS and that the C-terminal half of the NBS1 protein is essential for DSB repair. Our recent findings of complementation experiments using mutated cDNA also suggest that there may be an important domain in the C-terminus region of the NBS1 protein which expresses its functions (Tauchi et al, *in preparation*). This is consistent with the report of Carney et al (1998) that NBS1 interacts with hMRE11 at the C-terminal half of the protein[23].

At the N-terminal end, NBS1 has FHA and BRCT domains which are widely conserved in eukaryotic nuclear proteins related to the cell cycle, gene regulation, or DNA repair. Durocher et al (1999) reported that the FHA domain is a phospho-specific protein-protein interaction motif which recognizes phosphorylation of the target protein[45]. This means that NBS1 may function as a signaling protein although it does not have kinase activity nor known domains to transduce the signal. On the basis of our present knowledge, NBS1 seems to function as a regulatory factor in

DSB repair through interaction with hMRE11 at the C-terminal half of the protein. Other protein-protein interactions in the FHA or BRCT domains, however, may function in signaling DNA damage because these domains are conserved from yeast to humans. Further analysis using deleted mutants and the targeted gene should provide useful information about the function(s) of the NBS1 protein, and lead to a clearer understanding of the cellular response to ionizing radiation.

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