Expression pattern of the Nijmegen breakage syndrome gene, \textit{Nbs1}, during murine development

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The Nijmegen breakage syndrome (NBS; MIM 251260), is an autosomal recessive disease characterized by microcephaly, growth retardation, immunodeficiency and cancer predisposition. NBS cells show spontaneous chromosomal instability and hypersensitivity to ionizing radiation in combination with radioresistant DNA synthesis. At the cellular level, NBS has some features in common with ataxia telangiectasia. In this study the murine \textit{Nbs1} gene was used for an expression study in mouse embryos at different developmental stages as well as in adult mice. A low level of expression is observed in all tissues. Highly specific expression was observed in organs with physiologic DNA double strand breakage (DSB), such as testis, thymus and spleen. Enhanced expression is also found at sites of high proliferative activity. These are the subventricular layer of the telencephalon and the diencephalon, the liver, lung, kidney and gut, as well as striated and smooth muscle cells in various organs. In the adult cerebellum the postmitotic Purkinje cells are marked specifically. These expression patterns suggest that in addition to the role of the \textit{Nbs1} gene product as part of a DNA DSB repair complex, the \textit{Nbs1} gene product may serve further functions during development.

INTRODUCTION

Nijmegen breakage syndrome (NBS; MIM 251260) was first described 20 years ago by Dutch cytogeneticists (1,2). The disease presents as a combination of several symptoms among which growth retardation, microcephaly in combination with mild to moderate mental retardation, immunodeficiency and an increased incidence of malignancy are pathognomonic (3,4). The cellular phenotype is in some way similar to that described for ataxia telangiectasia (AT) (5), namely spontaneous chromosomal instability, fusion of telomeres as well as a high level of chromosome rearrangements involving chromosomes 7 and 14 at sites which harbour the immunoglobulin and T cell receptor genes in cultured B and T lymphocytes (6). Cultured lymphocytes and fibroblasts from NBS and AT patients show hypersensitivity to ionizing radiation (IR), including increased IR-induced chromosomal aberrations, a failure to halt semi-conservative DNA synthesis (radioresistant DNA synthesis) and to stop cells at the G1/S border (see refs 7,8 for reviews).

The \textit{NBS1} gene product, nibrin, has been found to be part of a complex which is a central player in the human cellular DNA damage response. In irradiated cells, the nibrin-containing protein complex becomes associated with double strand breaks (DSBs) and remains bound to these sites until DSB repair is complete (9). It is hypothesized that the complex functions in the detection of DNA damage. Further, the \textit{NBS1} gene product is involved in signal transduction for cell cycle checkpoint control after DNA damage. By cloning the \textit{NBS1} gene this assumption has now been partially confirmed. The \textit{NBS1} gene codes for a protein, nibrin, with 754 amino acids that contain a breast cancer C-terminal domain and a fork-head-associated domain (10), characteristic for proteins involved in cell cycle checkpoint control or DNA replication and repair. It is suggested that nibrin is the functional homologue of the yeast Xrs2, a protein involved in DSB repair (11,12). Actually, nibrin is involved in multiple signalling cascades which respond to DSBs induced by IR, or as part of normal processes such as meiotic recombination, mitotic V(D)J rearrangement in T and B lymphocytes and maintenance of telomere function (13,14). In addition, it is directly or indirectly involved in cell cycle checkpoint control and p53 activation (see refs 15–17 for reviews). Thus, since key regulatory processes are defective in \textit{NBS1} cells, a pleiotropic phenotype develops in affected patients. To understand the different functions of the \textit{NBS1} gene it was especially interesting to investigate whether \textit{NBS1} gene expression can be observed during development outside of tissues with well known physiologic DSB repair. Here we describe the pattern of \textit{Nbs1} gene expression during mouse development and compare this pattern with that described for the murine \textit{Atm} gene, \textit{Atm}.

RESULTS

Northern blot analysis

To determine the expression of the \textit{Nbs1} gene in mouse adult tissues northern blot hybridizations were performed with total RNA samples of different mouse tissues. The analysis revealed a single mRNA species of \(\sim 2.8\) kb (Fig. 1). High expression

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was found in the liver, heart and testis. Only low expression was visible in all other tissues analyzed.

**RNA in situ hybridization**

To determine the precise regions and the specific cells that express Nbs1 mRNA, in situ hybridization was performed using a 35S-labelled antisense RNA probe on whole-body embryo sections from 9.5–17.5 days post conception (d.p.c.) and specific sections derived from 2-day-old and adult mice.

**Fetal stage 9.5–12.5 d.p.c.** In the early mouse embryo [9.5 d.p.c. (Fig. 2a and b)] expression of Nbs1 is seen in all parts of the embryo, the highest expression is visible in the developing nervous system and the gut. Nbs1 is also expressed in the larynx part of the fetal placenta and in the extra-embryonic membranes, in particular in the visceral yolk sack. At 10.5 d.p.c., enhanced levels of expression are observed in proliferating cell populations (the matrixzone) of the forebrain, midbrain, hindbrain and spinal cord. At this stage Nbs1 mRNA was also detected in other organs. The expression is particularly prominent in the liver and in the maxillary component of the first branchial arch (data not shown).

In a 12.5 d.p.c. embryo (Fig. 2c and d), Nbs1 is expressed in various structures of the developing brain, e.g., in the subventricular layer of the lateral and fourth ventricles, in the thalamus and in the ganglionic eminence projecting into the third ventricle. The expression is restricted to cell layers with proliferative activity in the developing CNS, whereas no signals are visible over postmitotic mantle-zone neurons in the telencephalon. In the spinal cord, expression is restricted to the proliferating cells of the matrixzone. High expression is also found in the endoderm-derived organs of the respiratory and digestive tracts, in particular in lung, liver and gut. Expression signals are also seen in the muscle tissue of tongue and heart.

**Fetal stage 14.5–17.5 d.p.c.** During later prenatal development [14.5 d.p.c. (Fig. 2e and f)], expression of Nbs1 is highest in a variety of organs which are in the stage of organ expansion. Hybridization signals are prominent in the digestive tract. At this stage of development, the stomach and the liver occupy most of the peritoneal cavity, the midgut is still found in the physiological umbilical hernia. The pancreas consists of numerous secondary branchings which develop from the two original endodermal outgrowths at the foregut–midgut junction. These epithelial branchings extend into the surrounding mesenchyme. The epithelium itself is marked by Nbs1 expression.

Dentition is characterized by interactions between epithelium and the neural-crest-derived mesenchyme of the first branchial arch. The tooth bud is formed by rapid cell proliferation of mesenchymal cells on the basal surface of the dental lamina. The latter site is the area of Nbs1 expression during dentition (Fig. 2e and f). The bronchi and bronchioli of the outgrowing lung have numerous ramifications and end as alveoli. The epithelial cells as well as the surrounding mesenchymal cells proliferate and express Nbs1. High Nbs1 expression is observed in the thymus. Muscle tissue is also a site of Nbs1 expression. We found strong signals in the transverse striated muscle fibres of the tongue (Fig. 2c–f). The myocardiawalls of the atrial and ventricular chambers are also positive. The skeletal muscle (e.g., intercostal muscles near the sternum) and the smooth muscle cells in the wall of the urinary bladder (Fig. 2f) show a strong hybridization signal, too.

For comparison, a section of a 14.5 d.p.c. embryo hybridized with an RNA probe specific for Atm is added (Fig. 2h). In the large organs (brain, lung, liver and kidney) the expression patterns of both genes, Nbs1 and Atm, are very similar. But Nbs1 is also expressed in smooth and striated muscle tissues of the gut, bladder, heart and tongue and in mesenchymal tissue condensations which surround the vertebrae and model the upper and lower jaw (Fig. 2f). Therefore the appearance of the Nbs1 expression pattern is far more distinctive and differentiated than that of Atm (Fig. 2f).

At this time of development Nbs1 expression in the brain is restricted to very specific sites only. The cerebellum showed significant levels of Nbs1 expression within developing structures, including the granule layer (Fig. 3a and b). The surrounding structures (tegmentum, medulla and the inferior colliculus) also revealed Nbs1 expression. In mice, much of cerebellar maturation occurs during the first postnatal week. Therefore, we investigated Nbs1 expression in brains derived from 2-day-old and adult mice (Fig. 3c–f). Two days after birth expression was visible in the internal and the external granule layer. The external granule layer is divided into two functional zones: the proliferative and the premigratory zones. Nbs1 expression is detectable in cells of the proliferative zone only (Fig. 3e and d). In the adult cerebellum, Nbs1 transcripts are present in the granule cell layer (stratum granulosum) as well as in the Purkinje neurons (stratum ganglionosum). No expression was detectable in stratum molecare cerebelli (Fig. 3e and f).

Whereas Nbs1 expression is high and ubiquitous in the embryonic brain, it is comparatively low and limited to certain structures in the adult brain. In the adult brain the highest transcript levels were seen in the hippocampus (data not shown) and the cerebellum (Fig. 3e and f).

In late embryogenesis, expression of Nbs1 mRNA becomes low in most tissues. There is, for example, no further expression in the endoderm-derived organs of a 17.5 d.p.c. embryo. Expression continues in the proliferating zone of the kidney (data not shown).
Adult animal. In the adult animal the brain was analysed as was mentioned before. Further, the tissues with physiological DSB repair (testis, spleen and thymus) were tested (Fig. 3g–l). In the testis, the highest level of $Nbs1$ expression was observed in the seminiferous tubules of the mature testis (Fig. 3g and h). The expression is found in the seminiferous tubules of stages IX–XII of the gametogenic maturation cycle (18). No specific hybridization signal was detectable in the outermost cell layer of spermatogonia and in the postmeiotic spermatids and mature sperm cells in the inner lumen. High expression is confined to primary spermatocytes of the preleptotene to zygotene stage, which are present in the tubuli only during stages IX–XII, whereas pachytene cells are present throughout all stages (19).

The spleen is divided into two compartments containing specialized cell types. B lymphocyte maturation occurs in the germinal centres of the white pulp. The red pulp consists of reticuloendothelial cells and some types of macrophage and dendritic cell. In the adult spleen only weak expression was found by northern blot analysis (Fig. 3). RNA in situ hybridization reveals a highly specific signal of only some cells in the red pulp (Fig. 3i and j). The thymus is responsible for T lymphocyte differentiation. The organ is composed of cortex and medulla. In the cortex a stroma of epithelial reticular cells supports the maturation of T lymphocytes and secretes thymic factors essential for T lymphocyte maturation. Final maturation and negative selection of T lymphocytes occurs in the medulla. Specific and high signals of $Nbs1$ are present on single cells within the medulla only (Fig. 3k and l).

**DISCUSSION**

The phenotype of NBS patients is characterized by a specific combination of symptoms which are due to the pleiotropic effects of the $NBS1$ gene. To understand the function of this pleiotropic effect the sites of expression during development are described in this report.

Vissinga et al. (20) have previously reported first expression data for $Nbs1$ in different mouse tissues by northern and dot blot analysis. On a background level of steady-state $Nbs1$ transcription a several-fold variation, highest in testis, was found. A background level of expression was observed in all stages analysed during this study (Fig. 1; compare Fig. 2f and g). But on this background level of expression a specific pattern became apparent.

At stage 9.5 d.p.c. the whole embryo together with the extraembryonic tissues shows $Nbs1$ expression (Fig. 2a and b). Enhanced levels of signal are present at sites of high cellular
density in the epithelia of the neural tube and the digestive tract.

After completion of organogenesis, when the embryo enters the stage of histogenesis with organ enlargement, the expression of *Nbs1* becomes more and more distinct (Fig. 2c–f). In general, *Nbs1* expression marks regions of high proliferative activity. This is evident at stages 12.5 and 14.5 d.p.c. in the brain, liver, kidney and gut. For these large organs there is nearly complete overlap of expression for *Nbs1* and *Atm* (Fig. 2f and h).

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**Figure 3.** Expression of *Nbs1* in the developing mouse cerebellum and in some adult organs. (a, c and e) Brightfield and (b, d and f) darkfield images of the developing cerebellum at 14.5 d.p.c. (a and b), postnatal day 2 (c and d) and adult (e and f) hybridized with the *Nbs1* antisense riboprobe. ce, cerebellum; egl, external granule layer; gl, granule layer; igl, internal granule layer; ic, inferior colliculus; med, medulla; pl, Purkinje cell layer; teg, tegmentum. (g) Brightfield and (h) darkfield images of a section through the testis of an 8-week-old mouse. High *Nbs1* expression is visible in distinct seminiferous tubules (stages IX–XII) and lower expression in stage I–VIII tubules. No expression is detectable in the spermatogonia and in postmeiotic spermatids. (i and j) Sections through the spleen of an 8-week-old mouse. Specific cells of the red pulp (rep) show a high signal, whereas the germinal centres in the white pulp (whp) are not labelled. (k and l) Sections through the thymus of an 8-week-old mouse, showing expression in specific cells of the medulla (med) and cortex (cor).
Soares et al. (21) have examined the expression of Atm in the developing cerebellum very carefully. Comparable sections of the cerebellum at different developmental stages as provided (21) are shown for Nbs1 expression in Figure 3a–f. There is nearly a complete overlap of both patterns. Atm and Nbs1 are expressed in the primordia of the cerebellum at 14.5 d.p.c. (Fig. 3a and b) and become confined to the proliferation zone in the external granular layer of the cerebellar folia in the postnatal cerebellum (Fig. 3c and d).

But there are also some differences between the Atm and Nbs1 expression patterns. Most obvious is the expression of Nbs1 in muscle and connective tissue sheets, where Atm is not expressed or is too low to be detected. This difference in expression is responsible for the difference in appearance of both expression patterns during development (Fig. 2f and h). From the expression pattern observed Nbs1 seems to be more strictly linked to cellular proliferation than is Atm. Another difference becomes evident in the adult cerebellum. The highest Nbs1 activity is found in the large Purkinje cells which do not grow out from the granule cell layer before birth (Fig. 2f). Atm is not expressed in Purkinje cells. In adult animals Atm is expressed in sensory neurons of the dorsal root ganglia (21), where Nbs1 was found not to be expressed during this study. These cells are responsible for the afferent conduction of sensory stimuli to the Purkinje cells and the cerebellum. One of the main symptoms in AT patients is ataxia which is thought to be due to disturbed afferent conduction to the cerebellum. NBS patients do not develop ataxia, although Nbs1 was found specifically expressed in Purkinje cells in the brain of the adult mouse (Fig. 3e and f). Purkinje cells as well as the sensory neurons in the dorsal root ganglia are postmitotic cells. This observation suggests that in these cells Nbs1 and Atm serve another, as yet unknown, function which is different from signal transduction during proliferation and DSB repair.

It should be added here that the expression of Nbs1 and Atm in the cerebral cortex, hippocampus and cerebellum is very similar to those described for other genes with a function in DSB repair, such as the recombinase activating gene 1, RAG1 (22), and DNA-PK, which codes for a nuclear serine/threonine protein kinase required for DSB repair within a distinct end-joining pathway (23).

The function of Nbs1 during DSB repair is reflected by the specific expression profile of Nbs1 in the mature testis, spleen and thymus (Fig. 3g–l). In the mature testis, Atm expression has been localized along the synapsed chromosome axis in the zygotene and early pachytene stage of meiosis I by immuno-histochemical techniques (24,25). Nbs1 expression is found highest in tubuli seminiferi of stages IX–XII of the spermatogenic maturation cycle (Fig. 3g and h), and only during these stages do spermatocytes pass through leptotene and zygotene to early pachytene of the meiotic prophase (18,19). As shown here, the timing of Atm and Nbs1 expression during spermatogenesis proves to be the same. AT as well as NBS female patients show ovarian failure. Male AT patients prove to be infertile too. No firm data on fertility are available for male NBS patients (8).

The pattern of Nbs1 expression in the spleen (Fig. 3i and j) is difficult to explain. Since V(D)J recombination and immunoglobulin class switching of the B lymphocytes require the processing of DSBs, one would have expected a positive signal in the germinal centres of the white pulp. But it is evident that single cells of the red pulp bear a high and specific signal. These cells could be dendritic cells which are engaged in antigen recognition or macrophages. This is in some way a paradoxical expression pattern for a DSB repair gene in spleen and is also observed for the DNA-PK gene, which is observed in some reticuloendothelial cells of the red pulp (23). Also in the thymus a small subtraction of cells in the medulla show a distinct and high signal (Fig. 3k and l). Further cell fractionation studies of these organs will show which cells are responsible for high Nbs1 activity.

On the cellular level, the DSB defect in cells from NBS and AT patients has attracted most attention in the past. But NBS and AT patients show a broad spectrum of symptoms which can only be explained by more pleiotropic effects which these genes exert during development. This expression study provides evidence that apart from sites of physiologic DSB repair in the testis, thymus and spleen, Nbs1 (and also Atm) expression is present in several tissues and organs which are not known to undergo DSB repair. Therefore these genes may serve further, hitherto unknown, functions during development; for example: (i) an unknown function during cellular proliferation in certain tissues (brain, muscle, intestine) which might explain microcephalie due to small brain volume, facial dysmorphism and general growth retardation; or (ii) a further function in mature Purkinje cells (Nbs1) or afferent neurons of the dorsal root ganglia (Atm), which in AT patients leads to ataxia.

**MATERIALS AND METHODS**

**Northern blot analysis**

A 379 bp fragment, corresponding to bp −49 to +330 of the Nbs1 mouse cDNA, was random primed with 32P and hybridized to a mouse multiple northern blot (Clontech, Palo Alto, CA) according to the manufacturer’s instructions. The same blot was tested for β-actin expression as control.

**Mice**

Embryos were isolated from pregnant C57Bl/6 mice at the time indicated in the text. The day of plug detection was considered to be 0.5 d.p.c.

**RNA in situ hybridization**

For Nbs1 the full-length cDNA was used as a probe (20). The Atm probe was prepared by PCR on cDNA prepared from 14.5 d.p.c. whole embryos. The amplified sequence represents the murine Atm cDNA sequence (GenBank accession no. AF076687) from 8103 to 9026 bp. To the reverse primer was added the T7 RNA polymerase promoter which allows to synthesize directly antisense RNA from the PCR product (26). The identity of the probe was proven by DNA sequencing. RNA in situ hybridization was performed on whole-body embryo sections from 9.5–17.5 d.p.c. and specific sections derived from 2-day-old and adult mice. The embryos were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline at 4°C and prepared for cryostat sectioning. Sections
(10 µm) were mounted on slides. RNA in situ hybridization was carried out as described (27) with minor modifications.

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REFERENCES