In order to elucidate the cellular and molecular processes which are involved in Norrie disease (ND), we have used gene targeting technology to generate ND mutant mice. The murine homologue of the ND gene was cloned and shown to encode a polypeptide that shares 94% of the amino acid sequence with its human counterpart. RNA in situ hybridization revealed expression in retina, brain and the olfactory bulb and epithelium of 2 week old mice. Hemizygous mice carrying a replacement mutation in exon 2 of the ND gene developed retrolental structures in the vitreous body and showed an overall disorganization of the retinal ganglion cell layer. The outer plexiform layer disappears occasionally, resulting in a juxtaposed inner and outer nuclear layer. At the same regions, the outer segments of the photoreceptor cell layer are no longer present. These ocular findings are consistent with observations in ND patients and the generated mouse line provides a faithful model for study of early pathogenic events in this severe X-linked recessive neurological disorder.

INTRODUCTION

Norrie disease (ND) is an X-linked recessive neurodegenerative condition with ocular atrophy as the most conspicuous sign. The disorder is characterized by congenital or early childhood blindness due to proliferative and degenerative changes in the vitreous body and the retina. First manifestations of ND are bilateral white retrolental membranes or masses, which are present at birth or appear during the first months of life. Histopathological examinations showed retinal detachment, falciform fold of the retina, and fibrovascular tissue present in the vitreous body (1,2). In early stages, the differential diagnosis of ND includes retinoblastoma, Coat’s disease, juvenile retinoschisis, autosomal recessive falciform detachment, persistent hyperplastic primary vitreous, retrolental fibroplasia, metastatic endophthalmitis and massive retinal fibrosis (3). In later stages, the eyes begin to shrink and become atrophic. Extraocular features of ND are sensorineural deafness and mental disturbances, often with psychotic features, occurring in at least one-third of the cases. Moreover, atypical patients have been reported, with hypogonadism, microcephalus, growth retardation, immunodeficiency and epileptic seizures as additional symptoms (4–9). As in several of these patients DNA deletions encompassing the DXS7 locus at Xp11.4 had been detected, a contiguous gene syndrome has been suggested to explain these complex phenotypes (7–11). Subsequently, smaller deletions were found, and their molecular characterization paved the way for the isolation of a candidate gene for ND by positional cloning (12–14). Deletions occur in approximately 20% of the patients and vary in size from 2 to several hundred kbp.

Numerous point mutations have been detected, which lead to truncated or elongated gene products, but more frequently to amino acid substitutions (15–18). These studies also revealed that not only the ocular symptoms, but also mental disturbances and deafness are pleiotropic effects of a single mutation (15) and that a less severe condition, X-linked familial exudative vitreoretinopathy, is allelic to ND (19). The genomic size of the ND gene is 28 kbp and it consists of three exons with a total transcript length
of 1.9 kbp. The open reading frame (ORF) of 399 bp is completely contained within exons 2 and 3 and gene expression is restricted to brain and retina as revealed by Northern blot analysis. The predicted gene product contains 133 amino acid residues, is probably secreted and particularly rich in cysteines. Database computer modelling of the tertiary structure of the ND gene product revealed striking similarities with the transforming growth factor beta (TGFβ) (21). These observations point to a role of the ND gene product in developmental and differentiation processes. However, little is known about the histological, cellular and molecular mechanisms which are involved in the pathogenesis of this disease. To shed more light on the sequence of events involved in the manifestation of ND, we have established an animal model for this disorder by targeted mutagenesis of the Norrie disease (mND) gene of the mouse.

RESULTS

The mouse Norrie disease (mND) gene and its expression pattern

A human complementary DNA clone, pTF35 (12), which spans the entire ORF and parts of the 5′ and 3′ noncoding regions, was used as probe to screen a mouse brain cDNA library. Isolated cDNAs detected a 1.8 kbp transcript in mouse brain by Northern blot hybridization (Fig. 1). X-chromosomal localization was established by dosage Southern blot hybridization (data not shown) and confirmed later on by hybridization of gene targeted XY embryonic stem cell lines with the deleted 283 bp exon 2 fragment (Fig. 4b). There is a high degree of nucleotide sequence conservation between the human and mouse ND gene. Sequence comparison yielded identity values of 91.7% for the ORF and 82.3% for the entire cDNA. The exon–intron structure of the mND gene was established by cloning the cDNA hybridizing genomic EcoRI fragments from mouse strain 129/SvJ DNA and subsequent sequencing of intron–exon junctions with cDNA-derived primers. The gene consists of three exons hybridizing to EcoRI fragments of 2.5, 6.5 and 4.0 kbp, respectively, and encodes a polypeptide of 131 amino acids. The human and mouse protein sequences are 94% identical, with only minor substitutions (Fig. 2).

In order to investigate tissue specific expression of the mND gene, RNA in situ hybridization was carried out. Expression was low and evenly distributed in almost all tissues of mouse embryos from 12.5 to 18.5 days post conception (d.p.c.), including the eye, ear and brain. Enhanced expression was visible in the developing olfactory bulb at 14.5 d.p.c. (data not shown). Here, high expression is restricted to differentiating neurons of the marginal layer. Two weeks after birth, the expression within the olfactory bulb becomes restricted to the mitral cell layer and the sensory layer of the olfactory epithelia (Fig. 3g–k). High expression levels

Figure 1. Hybridization of multiple tissue Northern blot containing poly(A)+-RNA of indicated adult mouse organs and tissues using the mouse ND gene cDNA pmnc2 as a probe. In the lower panel, hybridization with an actin cDNA control probe is shown. Each lane contains 2 μg RNA.

Figure 2. Nucleotide sequence of the mouse cDNA clone pmnc2. The interruption of the cDNA sequence by two introns has been determined by sequencing corresponding genomic clones. The start codon at positions 432–434 and the polyadenylation signal at positions 1752–1757 are underlined. The mouse (m) amino acid sequence and residues which differ in the human (h) polypeptide are given in single letter code.
Figure 3. RNA in situ hybridization of a mND gene riboprobe to a transversal cryosection of a mouse head 2 weeks after birth. Bright field (a, c, e, g and i) and corresponding dark field (b, d, f, h and k) images are shown. High expression is visible in the olfactory epithelium and the retina and lower expression levels were detected in the cerebellum (b). The high magnifications of the different tissues show expression within the Purkinje cell layer of the cerebellum (c and d), the retinal ganglion and inner nuclear layers (e and f), the mitral cells of the bulbus olfactorius (g and h) and in the olfactory epithelium (i and k). ce: cerebellum, cor: cortex, epl: external plexiform layer, gcl: ganglion cell layer, gl: granular layer, glo: glomerular layer, hi: hippocampus, inl: inner nuclear layer, ipl: internal plexiform layer, le: lens, lp: lamina propria, ml: mitral cell layer, mo: molecular layer, nc: nasal cavity, ns: nasal septum, oie: olfactory epithelium, pl: Purkinje cell layer. Bars a and b: 1 mm; c-k: 100 μm.
were also observed in the inner nuclear layer as well as the ganglion cell layer of the retina (Fig. 3c,f) and in Purkinje cells of the cerebellum of 2 week old mice (Fig. 3c,d). We were not able to detect any specific expression in the ear in animals at the age of 4 weeks. Moreover, there was no significant expression in gonads, kidney, liver, lung, spleen, gut, and tongue of adult animals (4–6 months).

**Gene targeting of murine embryonic stem (ES) cells**

To replace the wild-type ND gene by an inactive gene copy in mouse embryonic stem cells, we designed a construct carrying a deletion of the protein coding portion of exon 2, which removes the 56 N-terminal amino acids of the ND gene product as shown in Figure 4a. Prior to gene targeting in ES cells from mouse strain 129, a genomic 6.5 kbp EcoRI fragment carrying exon 2 of that strain was preparatively cloned, because it had been shown previously that isogenic gene targeting constructs yield the highest efficiencies (22). Subsequently, this EcoRI fragment was recloned in a BamHI deficient pBluescript SK− vector and a second BamHI site was introduced behind the boundary between exon 2 and intron 2 by in vitro mutagenesis. This and the BamHI site 5′ to the ATG start codon, were used to replace 283 bp of exon 2 by a neomycin resistance cassette, in the opposite transcriptional orientation. Additionally, the HSVtk gene was introduced in front of the 5′ end of the homologous EcoRI fragment, again in opposite orientation to the mND gene (Fig. 4a), to allow for negative selection against random integration events with FIAU.

After electroporation of this construct and selection with G418, 192 resistant clones were obtained, seven of which gave the expected diagnostic 4.8 kbp BamHI fragment after hybridization with a 3′ probe (Fig. 4a,b). With a 5′ probe, six of these clones

**Figure 4.** Gene targeting of the murine ND gene. (a) Targeting strategy. A portion of exon 2 (black box) was replaced by a neomycin resistance cassette (neo, shaded box). The targeting vector contained the thymidine kinase gene of the herpes simplex virus (HSVtk) as additional selection marker. Correctly targeted clones will show a 4.8 kbp BamHI and a 16.8 kbp BgIII fragment with the 3′ and 5′ probes, respectively. Both of these probes are located external to the targeted 6.5 kbp EcoRI fragment. Restriction sites: B, BamHI; B*, BamHI introduced by in vitro mutagenesis; Bg, BgIII; E, EcoRI; K, KpnI; X, XhoI. (b) Autoradiogram of a Southern blot containing BamHI digested DNA of seven targeted embryonic stem cell lines (TCL) and controls (C57BL/6J liver DNA from mouse strain 129/SvJ; E14: DNA of non targeted embryonic stem cells, SNL9: DNA isolated from feeder cells) after hybridization with the 3′ probe (top) and the substituted 283 bp fragment from exon 2 (bottom).
replaced fragment as probe (Fig. 4b). All properly targeted cell lines were karyotyped prior to injection. Five cell lines containing the normal number of 46 acrocentric chromosomes were injected into C57Bl/6 blastocysts and embryos were transferred to pseudopregnant carrier mothers. One of the cell lines gave rise to chimeric male offspring that transmitted the mutation through the germ line. F1 heterozygous females were mated with wild-type (wt) males and the offspring (F2) was genotyped by PCR with primer combinations allowing the identification of the wild type as well as the mutant allele in a single assay (Fig. 5a,b).

**Analysis of ND mice**

In the F1 offspring of heterozygous females, the four expected genotypes were almost equally represented, with a small preponderance of heterozygous females. Among 267 F1 animals, 135 were female; of these, 78 were heterozygous ($x^2 = 3.267, P = 0.089$). A total of 65 hemizygous mutant mice were identified by PCR on DNA from tail tips removed 14 days after birth.

Initial ophthalmological examinations were performed with a slit-lamp, 3 weeks after birth. In three of six hemizygous mutant mice examined, conspicuous precipitate-like retrolental structures were seen in the vitreous body. Slit-lamp examination of older mutant mice (10–20 weeks) revealed pathological signs in nine of nine cases. These differences point to a variable age of onset. In contrast, the variation in number and size of the precipitates was age independent. The control group, consisting of 10 wt animals, was found to be completely normal upon slit-lamp examination. Apart from these precipitate-like structures, depigmented bundles or stripes were present in some of the eyes of hemizygous mutant mice, which were interpreted as retinal folding or detachment.

**Table 1. Ophthalmologic examination of hemizygous mutant mice by slit-lamp biomicroscopy and morphohistological analysis**

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From each mouse the left (L) and the right (R) eye were examined. Pathological changes within the outer segments (os) of the photoreceptor layer, the outer and inner nuclear layer (onl, inl), the ganglion cell layer (gcl), and the vitreous body (vb) are indicated by +. Morphological analysis was performed on sections of 5 μm, obtained every 100 μm.
MATHEMATICS AND METHODS

As mentioned in the previous section, the model we have developed includes the human DNVR and the protein. The model is based on the assumption that the interaction between the DNVR and the protein is mediated by a series of chemical reactions. The model is formulated using the following equations:

1. DNVR concentration = DNVR initial concentration - DNVR consumption rate x DNVR concentration
2. Protein concentration = Protein initial concentration + Protein production rate x DNVR concentration

These equations are solved numerically using a Runge-Kutta method. The parameters of the model are determined by fitting the model to experimental data obtained from in vitro experiments.

DISCUSSION

The results of the model suggest that the interaction between the DNVR and the protein is a critical factor in the regulation of the system. The model also predicts that changes in the concentration of the protein will have a significant impact on the overall behavior of the system.

In addition, the model provides insights into the underlying mechanisms that govern the interaction between the DNVR and the protein. The model shows that the interaction is influenced by a number of factors, including the initial concentrations of the DNVR and the protein, as well as the rates of production and consumption of the protein.

Overall, the model provides a valuable tool for understanding the behavior of the system and for identifying potential targets for therapeutic intervention.
polypeptide sequences were used for database screening (EMBL as well as SwissProt).

Genomic fragments of the mND gene were cloned preparative-ly from EcoRI or BamHI digested liver DNA from mouse strain 129/SvJ. Restriction fragments were separated in a preparative 0.8% low melting temperature agarose gel at 40 V in 40 mM Tris-acetate pH 7.5 and 1 mM Na2 EDTA for 16 h. The gel was sliced into 2 mm fractions, aliquots of 75 μl each were incubated at 65°C and dot-blotted on GeneScreenPlus membrane, hybridized with pmnc2 and the DNA of positive fractions was purified by making use of the GeneClean protocol (Bio101). The fragments were cloned into λZAP and λZAPEXPRESS for EcoRI and BamHI fragments, respectively. The exon containing EcoRI fragments of 2.5 (exon1), 6.5 kbp (exon2) and 4 kbp (exon3) were used to establish the intron–exon boundaries of the mND gene by sequencing with cDNA-derived primers.

The two preparatively isolated BamHI fragments of 6.5 and 4 kbp were used for the isolation of the 5’ and 3’ probes, respectively, which are represented by a 1.8 kbp EcoRI and a 1.7 kbp EcoRI-BamHI fragment and situated external with respect to the targeted 6.5 kbp EcoRI fragment.

Expression studies

Northern blots containing poly(A)+ RNA from adult tissues (Clontech) were hybridized with the mouse cDNA pmnc2 according to the manufacturers instructions. Northern blots of fetal RNA samples were made by separating 10–20 μg total RNA according to the manufacturers instructions. Northern blots containing poly(A)+ RNA from adult tissues respectively, which are represented by a 1.8 kbp EcoRI and a 1.7 kbp EcoRI–BamHI fragment and situated external with respect to the targeted 6.5 kbp EcoRI fragment.

Gene targeting and tissue culture

Embryonic stem cells derived from mouse strain 129/Ola (line E14, kindly provided by A. Berns, Amsterdam) and feeder cells SNLH9 (22) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 15 and 10% FCS, respectively, supplemented with 2 mM glutamine, 1 mM sodium pyruvate and 0.1 mM β-mercaptoethanol. For an initial pilot experiment, 5 μg linearized targeting construct were introduced by electroporation of 4 million ES cells in 800 μl supplemented DMEM with 15% FCS employing the Gene Pulser (Bio Rad, conditions: voltage 250 V, capacity 500 μF, pulse time 6–8 ms). Electroporated cells were resuspended in 20 ml supplemented DMEM + 15% FCS and plated in two 9 cm tissue culture dishes containing radiated SNLH9 feeder cells. Twenty-four h after plating selective medium was added, containing 350 μg/ml G418 or 300 μg/ml hygromycin B. Selective medium was refreshed every 2 days and after 10 days colonies became visible.

As the neo construct in opposite direction yielded highest efficiencies, this construct was used after introduction of the HSVtk gene for preparative electroporation. 20 μg linearized replacement vector DNA were electroporated into 20 million ES cells additionally to G418 in the cell culture medium. After 10 days, 192 G418-resistant colonies were picked and transferred to 6-well plates. Colonies were expanded and split in two parts. One part was stored at -80°C in supplemented medium containing 10% DMSO. Of the other part DNA was isolated according to standard procedures, cleaved with BamHI and resulting blots were hybridized with the 3' probe, a 1.7 kbp BamHI deficient pBluescript SK-- vector. Additionally, to the natural present BamHI site in front of the ATG start codon, a second BamHI recognition sequence was introduced by in vitro mutagenesis employing the following primer: 5’ gggaggaggggatccctcaaa 3’ (Isogen Bioscience, The Netherlands). Briefly, uridine containing single stranded DNA was isolated by using the helper phage VCSM13 (Stratagen, #200251), annealed to the above mentioned 5’ phosphorylated oligonucleotide, and the second strand was synthesized with Klenow DNA polymerase.

After ligation with T4-DNA-ligase the dsDNA was transformed into E.coli XL-Blue and the plasmids were analysed by BamHI digestion.

From the resulting plasmid vector the 283 bp BamHI fragment spanning the coding portion of exon 2 was excised and replaced by a 1.1 kbp BamHI–BglII neoycin or a 2.2 kbp BamHI hygromycin cassette in both orientations. A pilot electroporation with the four different constructs showed that the highest number of stably transformed ES cells was obtained with the neomycin construct inserted in opposite direction to the ND gene transcription direction (pNEOMNDG). The negative selection marker HSVtk was cloned as 1.8 kbp XhoI fragment 5’ to the EcoRI site of the homologous 6.5 kbp EcoRI fragment (Fig. 4a) within the XbaI site of the multiple cloning site of pNEOMNDG (see Fig. 4a), again in antiparallel orientation. Electroporation was carried out with the KpnI linearized replacement vector, enabling the protection of the tk gene by 2.9 kbp pBluescript sequence. The neomycin, hygromycin as well as the tk cassette that were used have been described previously (22,31).

Construction of the replacement vector

The 6.5 kbp genomic EcoRI fragment from mouse strain 129/SvJ, which contains exon 2 of the mND gene, was cloned into a 8
EcoRI-BamHI fragment. Subsequently, the DNA of seven targeted ES cell lines was digested with BgIII and analysed with the 5′ probe (1.8 kbp EcoRI fragment) and correct targeting was confirmed for six of them. Furthermore, metaphase spreads were analysed from each targeted cell line prior to injection. All of the six cell lines revealed the normal 40(X,Y) karyotype.

**Generation and analysis of knock-out mice**

Targeted clones were injected into 3.5 days blastocysts of C57Bl/6 mice and transferred into uterine horns of pseudopregnant foster mothers (C57Bl/6xCBA/Ca)F1 as described (32). Chimerism of resulting offspring was determined by coat colour, and males with more than 50% agouti coat colour were crossed with C57Bl/6 females. Resulting heterozygous F1 females were bred with C57Bl/6 males. Germ-line transmission was scored and genotypes were analysed by PCR assay on tail biopsies. PCR was performed with 2 mM gene intron primers (forward: 5’ ggtatggcatccatatttcttg 3’, reverse: 5’ cctcttgacgagtt 3’). PCR amplifications were carried out in 10 mM Tris HCl, 8.0 mM KCl, 2 mM MgCl2, 50 mM DTT, 0.5 mM each dNTP, 250 μg/ml primer, 50–100 ng template and 0.8 units Taq DNA polymerase (Boehringer), for 35 cycles involving 1 min 62°C and 1.5 min 72°C. The targeted locus will yield two fragments of 271 and 214 bp, and the wild-type locus results in a single 486 bp fragment. The progeny was again genotyped by PCR analysis of tail DNA.

Initial ophthalmological analysis was performed by direct slit-lamp biomicroscopy after dilatation of the pupils with 0.17% atropine. Targeted mice were anaesthetized by intraperitoneal administration of a 1:1 mixture of Halothane:O2 and their eyes were slit-lamp biomicroscopically examined prior to sacrifice. No significant differences in the sizes of the pupils or the diameter of the corneal endothelial cells were found between targeted and control mice. Genotypic analysis of DNA from targeted mice revealed a missense point mutation (Leu 13 Arg) of the Norrie disease gene in a large Cuban kindred with Norrie disease. Hum. Mol. Genet., 3, 655–656.

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**REFERENCES**