Purkinje Cell Degeneration Associated with Erythroid Ankyrin Deficiency in *nb/nb* Mice

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Abstract. Mice homozygous for the *nb* mutation (Chromosome 8) have a severe hemolytic anemia and develop a psychomotor disorder at 6 mo of age. The *nb/nb* mice are deficient in erythroid ankyrin (Ank-1) but, until the present study, the role of Ank-1 and of Ank-2 (brain ankyrin) in disease genesis was unknown. In normal erythroid tissues, we show that two major transcripts are expressed from *Ank-1*, and one of these is also present at high levels in the cerebellum. By in situ hybridization and immunocytochemistry, Ank-1 localizes to the cerebellar Purkinje cells and, to a lesser extent, the granule cells. In *nb/nb* mice, *Ank-1* transcripts are markedly reduced in both erythroid and neural tissue, and *nb/nb* Purkinje cells and granule cells are nearly devoid of Ank-1. The neurological syndrome appears concurrently with a dramatic loss of Purkinje cells. *Ank-2* maps to Chromosome 3 and its expression is unaffected by the *nb* mutation. We conclude that Ank-1 is specifically required for Purkinje cell stability and, in its absence, Purkinje cell loss and neurological symptoms appear.

SPECTRIN is the major component of the erythrocyte cytoskeleton (for reviews, see Bennett, 1990; Carraway and Carraway, 1989). Erythrocyte strength, stability, and viscoelastic properties are directly related to spectrin content (Waugh and Agre, 1988; Chasis et al., 1988). Ankyrin provides the major link between this spectrin-based membrane skeleton and the erythrocyte plasma membrane through high-affinity binding sites for beta spectrin and the cytoplasmic domain of Band 3 (Bennett and Stenbuck, 1980). Binding of the peripheral membrane protein 4.2 to ankyrin stabilizes the attachment of ankyrin to the erythrocyte plasma membrane (Korsgren and Cohen, 1988; Rybicki et al., 1988). In view of the pivotal role ankyrin plays in linking spectrin to the plasma membrane, its importance in maintaining erythrocyte integrity is easily appreciated. Ankyrin-deficient hemolytic anemias have been described in both mice (normoblastosis, gene symbol *nb*) and humans (Hereditary spherocytosis-2, gene symbol SPH-1) (Bodine et al., 1984; Costa et al., 1990; Lux et al., 1990*a*; Hanspal et al., 1991).

Immunoreactive forms of ankyrin have been found in many tissues including the brain (Davis and Bennett, 1984), kidney (Drenckhahn and Bennett, 1987), liver and testis (Bennett, 1979), the eye (Allen et al., 1987), and at the nodes of Ranvier (Kordeli et al., 1990). Ankyrin also binds such integral membrane proteins as the Na^+K^+ ATPase (Morrow et al., 1989) and the voltage-dependent Na^+ channels (Srinivasan et al., 1988). The colocalization of ankyrin with these integral membrane proteins at restricted sites in kidney tubules and neurons suggests that ankyrin binds these proteins to the plasma membrane and is important in the establishment of membrane polarity (Bennett, 1990; Morrow et al., 1989; Srinivasan et al., 1988).

The exact number of genes involved in the production of the tissue immunoreactive ankyrins is not known. Two ankyrins have been cloned, sequenced, and mapped in the human. ANK1 is the major variety in the human erythrocyte cytoskeleton and maps to chromosome 8 in both the human (Lux et al., 1990*b*; Lambert et al., 1990) and the mouse (White et al., 1990). The major form of ankyrin found in the brain (ANK-2) shares many physical and biochemical properties with Ank-1 (Davis and Bennett, 1984). It is encoded, however, by a structurally distinct gene, ANK-2 (Otto et al., 1991), and maps to human chromosome 4q (Tse, W. T., personal communication). The present report establishes the location of *Ank-2* on murine Chromosome 3. Recent evidence suggests that the ankyrin present at the nodes of Ranvier represents a gene product distinct from ANK1 and ANK2 (Kordeli and Bennett, 1991). Whether the ankyrins found elsewhere are products of distinct genes or are alternatively spliced products of ANK1 and ANK2 is not yet known.

Mice carrying the normoblastosis mutation have provided a useful model for establishing the effects of ankyrin deficiency upon the red blood cell membrane (Bodine et al.,

1984; White et al., 1990). We have found that, in addition to the hemolytic anemia, these mice develop an age-related neurological syndrome characterized by a persistent tremor and an awkward gait. In this report, we establish the relationship between Ank-1, Ank-2 and the neurological symptoms noted in the severely anemic *nb/nb* mice. We have demonstrated that Ank-1 is present in the cerebellum of normal mice and localizes to the Purkinje and granule cells, determined that *Ank-1* transcripts and erythroid ankyrin are severely reduced in *nb/nb* tissues and discovered an extensive loss of mutant Purkinje cells with time. We have further shown that *Ank-2* maps to Chromosome 3 and its expression is unaffected by the *nb* mutation. Hence, we conclude that Ank-1, erythroid ankyrin, plays a vital role in the stabilization of murine Purkinje cells, as well as of erythrocytes, and that its role in humans with SPH-1 should be investigated.

Materials and Methods

Animals

All mice were produced in our research colony at The Jackson Laboratory which is fully accredited by the American Association for the Accreditation of Laboratory Animal Care. The *nb* mutation was maintained in the heterozygous state on both the WB/Re (WB) and C57BL/6J (B6) inbred strains. Homozygotes (WBB6Fl-*nb/nb*) for study were obtained by crossing WB and B6 proven heterozygotes (Bernstein, 1980). To remove the effects of hybrid vigor, B6 homozygous (B6-*nb/nb*) mice were obtained by intercrossing B6 heterozygotes. WBB6Fl-*sph/sph* mice with a heritable alpha spectrin deficiency but a normal ankyrin genotype (Bodine et al., 1984) and phenylhydrazine-treated (Sigma P7126, Sigma Chemical Co., St. Louis, MO) (Chiu et al., 1980) anemic B6-+/+ mice were used as controls. Phenylhydrazine treatment increased the peripheral blood reticulocytes to >95% and caused erythroid hyperplasia of the spleen.

Ankyrin Nomenclature

It has been agreed upon by the respective mouse and human gene nomenclature committees that ankyrin genes will be named according to the order in which they are mapped. The homologous genes in each species will be given the same number. However, slightly different conventions prevail in the use of italics and upper versus lower case letters in naming the mouse and human genes. As an example, the symbols for the first ankyrin gene mapped are *Ank-1* in mouse and ANK1 in human. In this report, we have chosen to utilize the murine gene symbol to designate the protein: Ank-1 for isoforms derived from the *Ank-1*/ANK1 loci on mouse and human chromosome 8 and Ank-2 for isoforms derived from the *Ank-2*/ANK2 loci on mouse Chromosome 3/human chromosome 4.

Immunoblot Analysis of Reticulocyte Ghosts

Hemoglobin-depleted ghosts were prepared from adult B6-+/+ and WBB6Fl-*nb/nb* mice by repeated lysis in 5 mM sodium phosphate, pH 7.6, containing 1 mM diisopropyl fluorophosphate, 1 mM Na_2 EDTA and 50 mM Ep475 (E-64C) (Tamai et al., 1981). The Ep475 was a gift from Dr. A. L. Goldberg (Harvard Medical School). SDS-PAGE was performed according to the method of Laemmli (1970) using a 5% stacking/10% running gel. Protein concentration was determined by the method of Lowry (1951). The separated proteins were transferred in a semi-dry transfer apparatus (Bio-Rad Trans Blot SD; Bio-Rad Laboratories, Oxnard, CA) to Immobilon P in 48 mM Tris-HCl, 39 mM glycine, pH 9.2, with 20% methanol. Ankyrin was detected using an affinity-purified anti-human erythrocyte ankyrin IgG kindly provided by Dr. Panos Savvides (Harvard Medical School) and horseradish peroxidase conjugated Protein G (Bio-Rad) as described previously (Peters et al., 1990).

Hybridization Probes

The probe used to detect *Ank-1* transcripts was a 4.6-kb cDNA (mAnk-1) isolated by Drs. Sam Lux and Robert White (Harvard Medical School) from a size-selected (>2.0 kb) anemic mouse spleen lambda gt11 cDNA library kindly provided by Dr. Ron Kopito (Department of Biology, Stanford Univ.). The mAnk-1 cDNA probe extends from the carboxy end of the 89-kD domain of erythroid ankyrin (Lux et al., 1990*b*; Lambert et al., 1990) into the 3′ untranslated region. *Ank-2* transcripts were detected using a 2.0 kb human brain ankyrin (hBrank-2) cDNA isolated from a human brain stem lambda gt11 cDNA library by Dr. Edward Otto (Otto et al., 1991). This clone is distinct, by sequence, from mAnk-1.

Northern Blots

Total cellular RNA was isolated from spleen, bone marrow, peripheral blood, and PBS-perfused brain by the acid guanidinium-thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Polyadenylated RNA (poly(A)+RNA) was isolated by oligo(dT)-cellulose chromatography (Aviv and Leder, 1972). RNA was fractionated by formaldehyde-agarose gels and transferred to nylon filters (Sambrook et al., 1989). Probes were ^{32}P-labeled by the random hexamer priming method (Feinberg and Vogelstein, 1983). Hybridizations were as recommended for Zetabind nylon filters (AMF CUNO, Meriden, CT) except that the SDS concentration was increased to 0.5%. After hybridization, filters were washed and exposed to Kodak XAR-5 film at −70°C with an intensifying screen.

Genetic Mapping

A TaqI restriction fragment length polymorphism detected by hBrank-2 was typed in 99 progeny from an interspecific backcross between the B6 and *Mus spretus* strains of mice (Nadeau, 1989; Botstein et al., 1980; Southern, 1975). Nylon filter blots of restriction enzyme-digested genomic DNA from the backcross progeny were provided by Ceci et al. (1990) and hybridized in our laboratory. Map distances were calculated as number of crossovers, divided by number of progeny tested, times 100, and reported as map units (mean ± SEM).

Immunocytochemistry and In Situ Hybridization

Mice (2–3 mo old) were anesthetized with avertin and perfused through the left ventricle with 20 ml PBS (Gibco 310-4200AG, pH 7.4; Gibco Laboratories, Grand Island, NY) followed by 20 ml cold (4°C) 4% paraformaldehyde in PBS. The cerebellum was removed and immersed in fresh 4% paraformaldehyde for 4 h at 4°C. The tissues were routinely processed to paraffin, cut at 8 μm, and mounted on baked (180°C overnight) poly-L-lysine-coated slides. Slides were deparaffinized, dehydrated, air dried, and stored dessicated at −70°C.

For immunocytochemistry, an antibody was prepared (VB) using the ANK-1 specific human sequence, which is spliced out to convert ankyrin 2.1 to activated ankyrin 2.2 (Lambert et al., 1990; Lux et al., 1990*b*). Three different peptides (15 residues each) corresponding to this ANK-1 specific insert were prepared and conjugated to glutaraldehyde-activated rabbit serum albumin and all three peptides were used for immunization. The antibody was affinity-purified on a human ankyrin 2.2 column to remove cross reactivity to other ankyrin epitopes and on a sheep ankyrin 2.1 column to obtain an antibody that both reacts with intact protein and cross-reacts with other species.

To detect ANK-1 in brain sections, endogenous peroxidase activity was blocked by incubating deparaffinized slides two times in 3% hydrogen peroxide in absolute methanol for 15 min each. Nonspecific protein binding was blocked by incubation in PBS containing 10% ovalbumin (Fisher A388-500) for 30 min. Slides were incubated with ANK-1 specific antibody diluted 1/250 in PBS containing 1% BSA (Sigma A7630) at 37°C in a humidified chamber overnight. After two washes in PBS, antibody binding was detected by the immunoperoxidase reaction using the ABC Vector system (PK4001; Vector Laboratories, Inc., Burlingame, CA).

To detect ankyrin messages, slides were incubated in proteinase K (2 μg/ml) (BRL Laboratories, Gaithersburg, MD) in PBS for 20 min at 37°C, washed in PBS and incubated overnight at 42°C in a humidified chamber in hybridization solution: 2× SSC (0.3 M sodium chloride, 0.03 M sodium citrate), 0.1 M sodium phosphate (pH 6.5), 1× Denhardt's, 10% dextran sulfate, 0.05% sonicated herring sperm DNA and 50% formamide with 500 ng/ml mAnk-1 probe biotinylated using the BRL Bio Nick system (8247SA). For controls, nick translated biotinylated pBR322 was used as the hybridization probe. The slides were rinsed twice in 2× SSC–0.1% SDS and 0.2× SSC–0.1% SDS at room temperature followed by two 15-min washes in 0.2× SSC–0.1% SDS at 50°C. The sections were rinsed in buffer (0.1 M Tris-HCL, pH 7.5, 0.1 M NaCl, 2 mM $MgCl_2$, 0.05% Triton X-100) and blocked for 20 min at 42°C in buffer containing 3% BSA. Biotinylated

probe was detected with streptavidin-biotin (alkaline phosphatase conjugate) and visualized with nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate using a commercial kit (BRL 8239SA).

Histopathology

Mice were anesthetized with avertin, perfused through the left ventricle with 20 ml of Bouin's fixative, and immersed overnight in 500 ml of Bouin's. The next day, the cerebellum was removed and routinely processed to paraffin. Serial sections were cut at 8 μm, stained with luxol fast blue, and counterstained with cresyl violet. Purkinje cell density was determined by counting the cells in random fields along the Purkinje cell layer using a calibrated ocular scale. Results were expressed as number of Purkinje cells per millimeter. After the Purkinje cell counts were completed, all fields were carefully examined to determine if any pathological changes restricted to particular anatomical sites within the cerebellum were present.

Results

The Ank-1 Protein Is Present but Markedly Reduced in nb/nb Cells

Previously, we showed by Western blot analysis that there are small amounts of a 150-kD erythroid ankyrin bound to the cell membrane of *nb/nb* reticulocytes but we were unable to detect the normal-sized 210-kD Ank-1 (White et al., 1990). By inclusion in the lysis buffer of the additional protease inhibitors, DFP and Ep 475, and by use of the more efficient semi-dry transfer system and enhanced protein binding to Immobilon P, we demonstrate the presence of 210 kD Ank-1 in *nb/nb* ghosts (Fig. 1, lane 2). By comparison with the normal Ank-1-reactive species in lane 1, it is clear that the mutant Ank-1 is markedly reduced and that the 150-kD Ank-1 is unique to the mutant adult reticulocytes. Kordeli and Bennett (1991) in a companion paper show by Western blotting that Ank-1 protein is present but markedly reduced in *nb/nb* brain, whereas Ank-2 is present at normal levels.

Multiple Transcripts of the Ank-1 Gene Are Expressed in Normal Erythroid Tissues and Are Deficient in nb/nb Erythroid Tissues

To establish the effects of the *nb* mutation on *Ank-1* mRNAs, we first compared the RNA species isolated from normal and *nb/nb* erythroid cells and tissues. Fig. 2 shows the results obtained when RNA filter blots of poly(A)+RNA from normal and *nb/nb* erythroid tissues are probed with ^{32}P-labeled Ank-1 cDNA (mAnk-1). Two transcripts, ~7.5 and 9.0 kb are detected in spleen and reticulocyte poly(A)+RNA from normal B6 mice with a phenylhydrazine induced anemia (lanes *1* and *3*). There are tissue specific differences in the relative amounts of 7.5 and 9.0 kb expressed. In spleen and bone marrow the ratio of 7.5 and 9.0 kb is ~2:1 (lanes *1* and *5*), whereas in reticulocytes only trace amounts of the 9.0-kb mRNA are expressed (lane *3*). In the *nb/nb* tissues, the 7.5- and 9.0-kb transcripts are greatly reduced (<25% of normal) (lanes *2* and *4*) or not detectable (lane *6*) by this method. Confirmation of the decrement in mutant mRNA species was established independently by quantitative dot blot methods (Sweetser et al., 1988; Thurston and Saffer, 1989) (data not shown). The *nb/nb* transcripts appear to be normal or near normal sized and to be expressed in the same relative proportions as the normal transcripts. Results show the reduction in mutant *Ank-1* mRNA levels correlates with the Ank-1 protein deficiency noted above in erythroid cells.

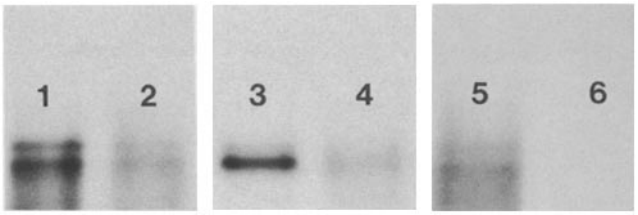

Figure 2. Autoradiograms of RNA filter blots hybridized to ^{32}P-labeled erythroid ankyrin probe, mAnk-1. Lane *1*, 5 μg B6-+/+ spleen poly(A)+RNA; lane *2*, 5 μg WBB6F1-*nb/nb* spleen poly(A)+RNA; lane *3*, 5 μg B6 +/+ reticulocyte poly(A)+RNA; lane *4*, 5 μg WBB6F1-*nb/nb* reticulocyte poly(A)+RNA; lane *5*, 10 μg B6-+/+ bone marrow total RNA; lane *6*, 10 μg WBB6F1-*nb/nb* bone marrow total RNA. Band sizes in kilobases were determined relative to 18 and 28S RNA.

Ank-1 Gene Transcripts Are Expressed in Normal Brain and Are Deficient in nb/nb Brain

To determine if the *nb* mutation has a similar effect on the brain, RNA filter blot analysis with Ank-1 and Ank-2 cDNA was performed. With Ank-1 cDNA (Fig. 3), a single transcript that comigrates with the 9.0-kb band in spleen RNA is detected (lanes *1* and *2*). This transcript is expressed at a much higher level in the cerebellum than in the rest of the brain (lanes *3* and *4*). As noted in *nb/nb* erythroid tissues, the level of the 9.0-kb transcript is greatly reduced in *nb/nb* cerebellum (lanes *5* and *6*).

Fig. 4 shows the three transcripts of ~10, 11, and 7.5 kb

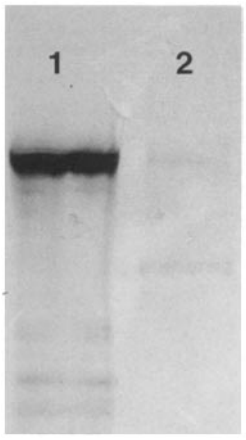

Figure 1. Immunoblot of hemoglobin-depleted ghosts (100 μg) from B6-+/+ (lane *1*) and WBB6F1-*nb/nb* (lane *2*) mice probed with anti-human erythrocyte ankyrin. In *nb/nb* mice, normal (210 kD) Ank-1 is severely reduced and a truncated 150-kD immunoreactive protein is present.

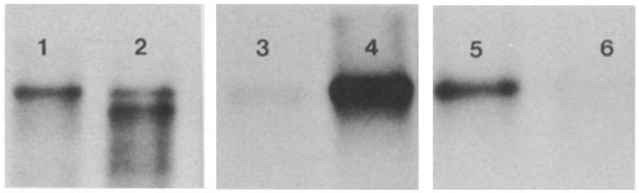

Figure 3. Autoradiograms of RNA filter blots hybridized to ^{32}P-labeled erythroid ankyrin probe, mAnk-1. Lane *1*, 10 μg B6 +/+ whole brain total RNA; lane *2*, 5 μg B6-+/+ spleen poly(A)+ RNA; lane *3*, 15 μg B6-+/+ whole brain minus cerebellum total RNA; lane *4*, 15 μg B6-+/+ cerebellum total RNA; lane *5*, 10 μg B6 +/+ cerebellum total RNA; lane *6*, 10 μg WBB6F1-*nb/nb* cerebellum total RNA. Band sizes in kilobases were determined relative to 18 and 28S RNA.

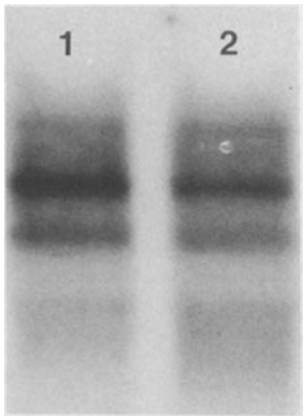

Figure 4. Autoradiograms of RNA filter blots hybridized to ^{32}P-labeled brain ankyrin probe, hBrank-2. Lane *1*, 15 μg B6-+/+ whole brain total RNA; lane *2*, 15 μg WBB6F1-*nb/nb* whole brain total RNA. Band sizes in kilobases were determined relative to 18 and 28S RNA.

detected with the Ank-2 cDNA probe (hBrank-2). Expression of *Ank-2* is normal in *nb/nb* brain (lanes *1* and *2*) as predicted by the results of Kordeli and Bennett (1991) and the mapping data (below).

Ank-2 Is Encoded by a Gene on Mouse Chromosome 3

To determine if *Ank-2* is linked to *Ank-1* on Chromosome 8, we mapped *Ank-2*. Assignment of *Ank-2* was done by analysis of 99 progeny from the interspecific backcross outlined in Table I. Nylon-filter blots of TaqI restriction enzyme-digested genomic DNA from backcross progeny were kindly provided by Ceci et al. (1990). Hybridization and RFLP analysis using the Ank-2 probe (hBrank-2) were done at The Jackson Laboratory. RFLP typing for *Amy-2* and *Egf* was provided by Ceci et al. (1990). The results of this analysis place the *Ank-2* locus on the distal end of mouse Chromosome 3, 7.0 + 2.5 map units distal to the well-defined marker locus *Amy-2* and 3.0 ± 1.7 map units proximal to the *Egf* locus. Human ANK2 has recently been mapped to chromosome 4 within the region q25-27 (Tse, W. T., personal communication). By mapping *Ank-2* to the distal end of mouse Chromosome 3, we have extended the known homology between Chromosome 3 and human chromosome 4q.

Ank-1 Is Present in Granule Cells and Purkinje Cells of the Normal Cerebellum but Is Markedly Reduced in nb/nb Cerebellum

Our hybridization blot analysis described above demonstrated a high level of Ank-1 mRNA in the normal cerebellum and severely reduced levels in *nb/nb* cerebellum. We further investigated Ank-1 distribution by in situ hybridization using biotinylated mAnk-1 cDNA as the probe. In normal cerebellum, Purkinje cells show intense positive signal (Fig. 5 *a*). Granule cells are also positive, although to a lesser extent. Interestingly, as has been reported previously for microtubule-associated protein mRNA (Tucker et al., 1989), hybridization signal is seen in the dendrites extending into the molecular layer indicating possible local synthesis of Ank-1. In *nb/nb* mice, the Ank-1 hybridization intensity in Purkinje cells is reduced to or near background levels, there is no evidence of Ank-1 in Purkinje cell dendrites, and Ank-1 in granule cells is clearly reduced (Fig. 5 *b*). Purkinje cells and their dendrites, as well as granule cells, are negative when biotinylated pBR322 is used as a probe (data not shown).

As a correlate to the in situ hybridization studies, we used antiserum specific for Ank-1 for immunocytochemical investigation of normal and *nb/nb* cerebellums. Intense positive signal is found in the cell bodies of Purkinje cells and within the granule cell layer of normal mice (Fig. 5 *c*). Diffuse staining within the molecular layer is also present, but whether this represents labeling of dendrites or axons (or both) can not be determined at this level. In *nb/nb* cerebellum, signal is markedly reduced in all structures (Fig. 5 *d*). Using this same Ank-1 specific antibody, these results have been confirmed by both tissue immunofluorescence and Western blotting in *nb/nb* brains (Kordeli and Bennett, 1991).

Table I. Genetic Linkage of Ank-2 on Chromosome 3

Chromosome inherited from F1 parent					
Amy-2		*Ank-2* $n = 99$		*Egf*	
B		B		B	54
S		S		S	35
B	x	S		S	3
S	x	B		B	4
B		B	x	S	1
S		S	x	B	2

Map distances
Amy-2 - *Ank-2* 7.0 ± 2.5
Ank-2 - *Egf* 3.0 ± 1.7

Cross: (C57BL/6J × *Mus spretus*)F1 × C57BL/6J.
Total progeny tested = 99. Gene order: Centromere–//–*Amy-2*–7.0–*Ank-2*–3.0–*Egf*–––.

A Progressive Loss of nb/nb Purkinje Cells Correlates with the Age-dependent Psychomotor Disorder

Having demonstrated that there are abnormal levels of *Ank-1* mRNA and protein in the mutant cerebellum, we sought pathological changes that would explain the appearance of the neurological syndrome in these mice. Purkinje cell density was determined from luxol fast blue/cresyl violet-stained serial sections of normal (B6), non-ankyrin-deficient but anemic WBB6F1-*sph/sph* and B6-*nb/nb* cerebellums. Representative sections are shown in Fig. 6. Purkinje cell size, morphology and number appear normal in *nb/nb* cerebellum at 6 wk of age (*a* and *b*). However, at 5–7 mo a striking loss of morphologically normal-appearing Purkinje cells is evident in B6-*nb/nb* cerebellum (*d*) when compared to age matched normal B6 and WBB6F1-*sph/sph* anemic controls (*c* and *e*).

Table II presents quantitative data on Purkinje cell density for control and *nb/nb* cerebellums from 1 to 12 mo of age. By 5–7 mo, the Purkinje cell density in *nb/nb* brains is ~50% of normal. No further loss is noted in mutant mice by 10–12 mo of age. The Purkinje cell loss in *nb/nb* mice occurs in random clusters and does not localize to any specific anatomical area of the cerebellum. The psychomotor disorder is apparent in all B6-*nb/nb* mice by 6 mo of age. This manifests itself in an awkward, unbalanced gait and a moderate tremor which persists even when the animal is at rest. Non-ankyrin-deficient anemic control mice (WBB6F1-*sph/sph*) do not exhibit neurological effects up to 12 mo of age.

Purkinje cell loss and the appearance of the psychomotor disorder are delayed relative to the Ank-1 mRNA deficiency. Ank-1 transcripts are already deficient in *nb/nb* cerebellum at 1 mo of age and there is no further change at 3 and 6 mo (mature adults) (Fig. 7) or up to 12 mo (data not shown).

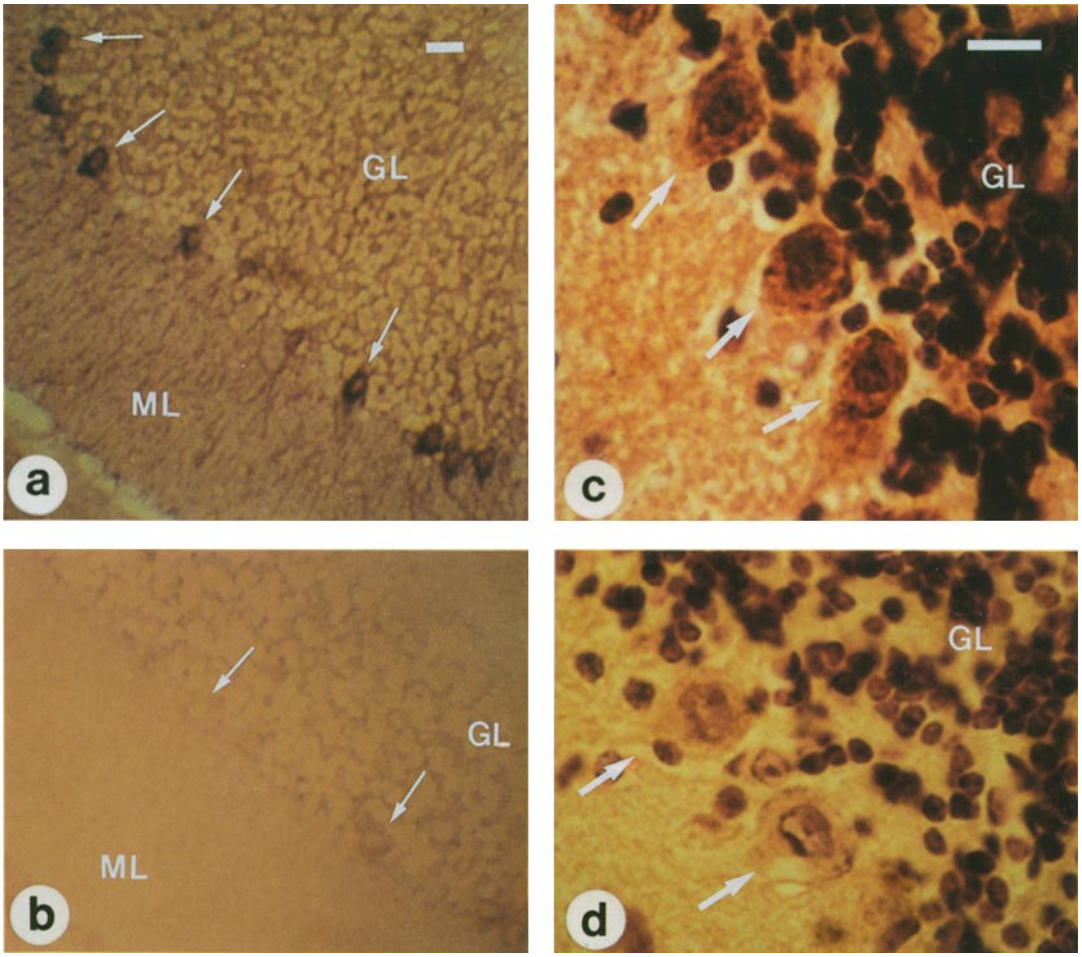

Figure 5. Detection of Ank-1 message in B6-+/+ (*a*) and WBB6F1-*nb/nb* (*b*) mice by in situ hybridization using biotinylated mAnk-1 as probe. Hybridization signal was detected with alkaline phosphatase. No counterstrain was used. Immunocytochemical demonstration of Ank-1 protein in B6-+/+ (*c*) and WBB6F1-*nb/nb* (*d*) cerebellum using human Ank-1-specific antiserum. Binding was detected with horseradish-peroxidase. Slides were counterstained with hematoxylin. The Purkinje cell layer (*arrows*) and, to a lesser extent, the granule layer (*GL*) of B6-+/+ mice contain abundant Ank-1 protein and Ank-1 mRNA. Diffuse reaction is also seen in the molecular layer (*ML*) of B6-+/+ mice. In *nb/nb* mice, Ank-1 protein and message are markedly reduced in all structures. Bars: (*a* and *b*) 50 μm; (*c* and *d*) 25 μm.

Discussion

This report describes a previously unobserved neurological component of the hemolytic anemia mutation, *nb*, and correlates Purkinje cell loss with the depletion of a specific structural protein, Ank-1. There is no apparent role of the major brain ankyrin, Ank-2, in the genesis of the disease. Abundant evidence exists that the *nb* mutation is a small gene lesion restricted to the *Ank-1* locus and, therefore, that the neurological sequelae in *nb/nb* mice are a direct consequence of Ank-1, erythroid ankyrin, deficiency. First, *nb* and *Ank-1* map to the same region of murine Chromosome 8 (White et al., 1990), and extensive restriction fragment length analysis has not detected mutation related restriction fragment length polymorphisms in *nb/nb* genomic DNA (data not shown). Second, normal sized Ank-1 protein is present but much decreased in the mutant reticulocytes. Third, low levels of Ank-1 mRNA, also of normal size, are detectable in *nb/nb* reticulocytes, erythro-generative tissues, and cerebellum. The absence of restriction fragment length polymorphisms and the presence of even small amounts of apparently normal sized Ank-1 protein and mRNA in *nb/nb* mice precludes the presence of a large chromosomal deletion, which potentially could affect other genes, in these mutants.

These data confirm the relationship between *nb* and *Ank-1*, but provide only partial evidence that the Ank-1 deficit is responsible for the neurological manifestations. The marked reduction of Ank-1 mRNA in the *nb/nb* Purkinje cells by in situ hybridization and of Ank-1 protein by immunocytochemistry are a more telling argument for the role of Ank-1 in the neurological disease. Kordeli and Bennett (1991) have recently completed an elegant immunofluorescent study of ankyrin protein distribution in normal and *nb/nb* neural tissue which confirm our findings concerning *nb/nb* Purkinje cells. These findings coupled with the dramatic loss of *nb/nb* Purkinje cells at the time disease symptoms first appear provide proof that Ank-1 is directly involved in the neurodegenerative disease. A possible role of the anemic state in the degeneration is precluded by the use of anemic but Ank-1 normal *sph/sph* mice as controls.

The interactions of Ank-1 with other cytoskeletal proteins in Purkinje cells are yet to be defined. Immunoreactive forms of spectrin have been localized to chicken and rat Purkinje cells (Lazarides and Nelson, 1983; Goodman and Zagon,

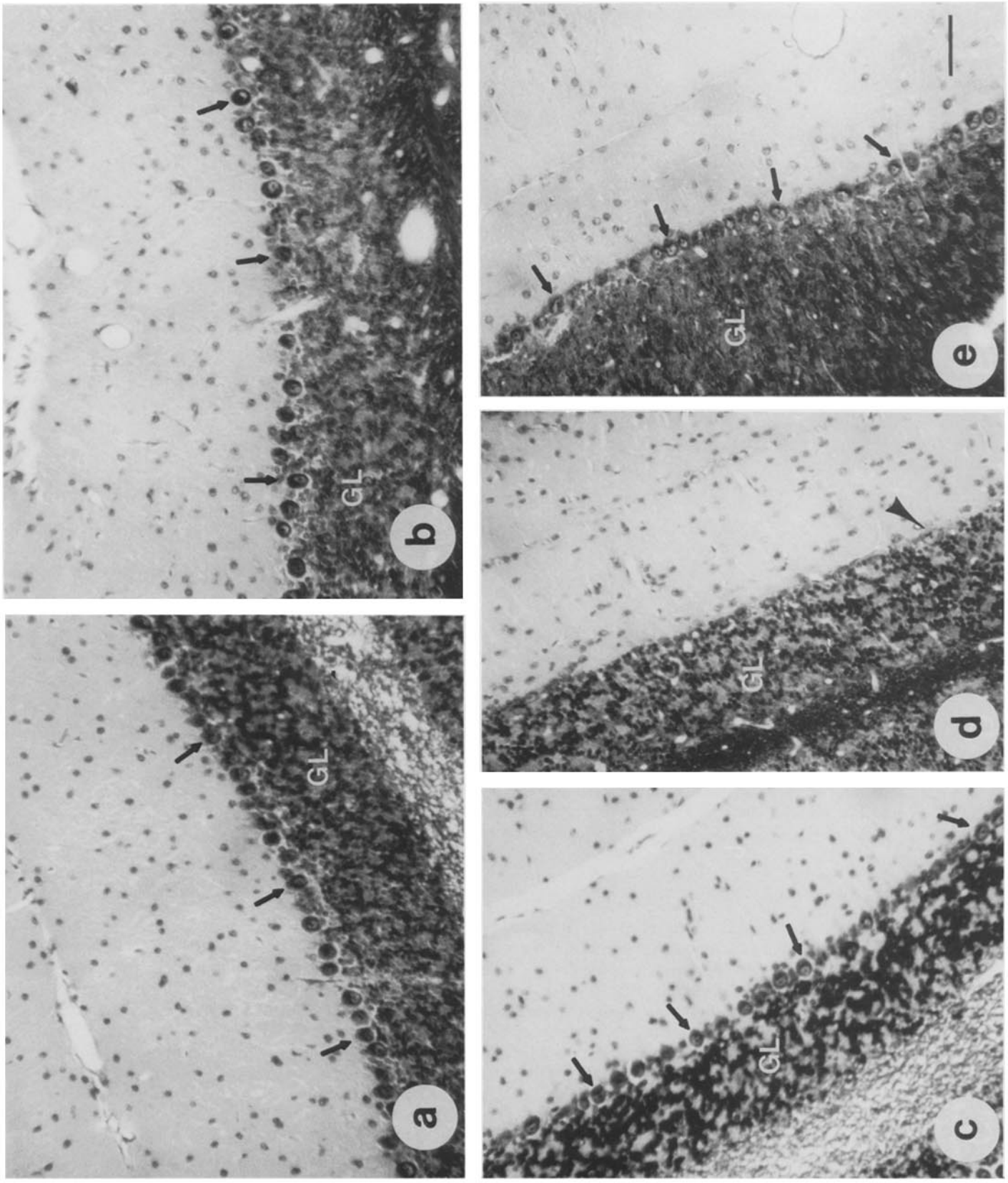

Figure 6. Luxol fast blue/cresyl-violet-stained tissue sections of normal and anemic cerebellums from mice of different ages. (*a*) B6-+/+ at 6 wk of age; (*b*) B6-*nb/nb* at 6 wk of age; (*c–e*) B6-+/+, B6-*nb/nb*, WBB6F1-*sph/sph* at 6 mo of age. The Purkinje cell layer is indicated with arrows, GL indicates the granule cell layer. Loss of Purkinje cells at 6 months of age is evident in B6-*nb/nb* mice (*d*) where only one apparent Purkinje cell is evident (*arrowhead*). Bar, 100 μm.

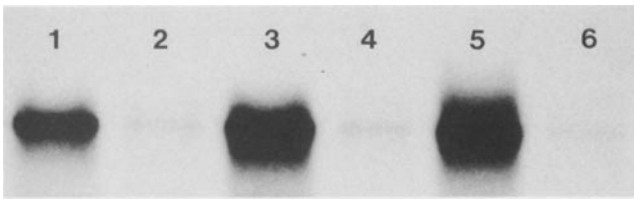

Figure 7. Autoradiogram of a cerebellar RNA filter blot hybridized to ^{32}P-labeled erythroid ankyrin probe, mAnk-1. Lanes *1*, *3*, and *5*, 15 μg total RNA from B6-+/+ cerebellums at 1, 3, and 6 mo of age; lanes *2*, *4*, and *6*, 15 μg total RNA from WBB6F1-*nb/nb* cerebellums at 1, 3, and 6 mo of age. Similar patterns are seen up to 12 mo of age (data not shown). Band sizes in kilobases were determined relative to 18 and 28S RNA.

1985), and an isoform of Band 3 is present in rat brain (Kay et al., 1991). In addition, we have demonstrated the presence of erythroid beta spectrin in normal mouse brain by Northern analysis (data not shown). Hence, potential interactions between Ank-1 and other membrane cytoskeletal proteins in Purkinje cells are numerous. It is likely that deficiencies in some of these proteins, particularly spectrin, contribute to Purkinje cell deterioration in *nb/nb* mice. Indeed, this is the case in *nb/nb* reticulocytes, which accumulate only 50% of normal spectrin levels (Bodine et al., 1984). However, *nb/nb* mice are congenic with their normal littermates and differ only at the *nb (Ank-1)* locus on Chromosome 8; reductions in spectrin, therefore, are a secondary consequence of the ankyrin deficit.

It is noteworthy that psychomotor disorders are found in human beings deficient in ankyrin (Coetzer et al., 1988) and in cases of ill-defined hereditary spherocytosis and "constitutional hemolytic icterus" (McCann and Jacob, 1976). In view of our findings, neurological sequelae in human beings with some forms of hereditary spherocytosis may be a direct consequence of Ank-1 deficiency. Of particular interest are the descriptions of patients with HS that, like *nb/nb*, develop neurological symptoms later in life which do not exacerbate with age (McCann and Jacob, 1976).

Table II. Age-related Purkinje Cell Density in Normal and Anemic Mice

	Purkinje cells/mm		
Genotype	1–3 mo	5–7 mo	10–12 mo
B6 +/+	38.3	32.6	32.9
	34.9	30	34.0
	36.3		31.5
	34.2		31.0
WBB6F1	35.7	32.6	34.6
sph/sph	36.4	36.9	30.3
	37.4		34.9
	35.9		
B6	35.7	16.2	15.3
nb/nb	33.2	13.5	15.7
	31.9		14.8
	32.3		

Each Purkinje cell density value represents the average number of Purkinje cells per millimeter determined for an individual mouse by counting the number of Purkinje cells in 100 random fields, 0.375 mm long.

Delayed onset of neurological symptoms is not an unusual phenomenon in heritable neurological syndromes. In 80% of mouse mutants with motor neuron degeneration (gene symbol, *Mnd*), motor neuron loss with onset of symptomatic behavior occurs at 7–8 mo of age (Messer et al., 1987). Symptoms of an autosomal recessive form of spinocerebellar degeneration in human kindred appear at an average age of 15 yr (Al-Din et al., 1990). Autosomal dominant Machado–Joseph Disease can appear in the fourth and fifth decade (Suite et al., 1986), while autosomal dominant adult amyotrophic lateral sclerosis appears at 19–46 yr (Veltema et al., 1990).

The neurological manifestations of the *nb* mutation are reminiscent of other murine mutations characterized by Purkinje cell loss. These include Purkinje cell degeneration (*pcd*), lurcher (*lc*), staggerer (*sg*), and nervous (*nr*) (Mullen et al., 1976; Wetts and Herrup, 1982; Herrup and Muller, 1979*a*; Wassef et al., 1987). In *pcd/pcd* mice, only 1% of the Purkinje cells remain by postnatal day 28, yet, as in *nb/nb* mice, only a moderate ataxia is seen and this only when Purkinje cell loss reaches 50% (Muller et al., 1976). In *pcd/pcd*, *sg/sg* and *nr/nr* mice, regional anatomical differences in Purkinje cell loss occur thus defining, by virtue of their response to the various mutations, subpopulations of Purkinje cells within the cerebellum (Herrup and Muller, 1976*b*; Wassef et al., 1987). The *nb* mutation is similar to these Purkinje cell loss mutants in that the mutation appears to affect a subpopulation of Purkinje cells. This subpopulation, however, is not confined to any specific anatomical area of the cerebellum but is, instead, in random clusters throughout the cerebellum.

Other sites where Ank-1 are diminished may be present in *nb/nb* mice since erythroid ankyrin is widely disseminated in the central nervous system, including the spinal cord and peripheral nerves (Kordeli et al., 1990). Hence, as is the case in human spinocerebellar degeneration, systems in addition to the cerebellum and its neuronal pathways are likely to contribute to the neurological symptoms of *nb/nb* mice.

In addition to characterizing the neurodegenerative effects produced by *nb*, these studies report, for the first time, the types and distribution of murine Ank-1 and Ank-2 mRNAs in the brain and erythroid tissues. Two *Ank-1* transcripts (~7.5 and 9.0 kb) are expressed in erythroid tissues of normal mice. Similar-sized transcripts have been reported in human erythroid tissues (Lambert et al., 1990). In the brain, a single 9.0-kb erythroid ankyrin transcript is present with expression highest in the cerebellum. The transcripts are also detected in RNA from erythroid and brain tissues of *nb/nb* mice; however, the level of the transcripts in each tissue is dramatically reduced. The sizes of the *nb/nb* transcripts are in the normal range, but we can not exclude the possibility that small insertions or deletions have occurred. These would be difficult to detect on filter blots because of the large size and low levels of transcripts detected. The common action of *nb* on both the erythroid and brain transcripts supports the conclusion that the 9.0-kb transcript in brain is a product of the *Ank-1* gene and not the result of cross-hybridization to a closely related message. In addition, the data suggest, by the same argument, that the multiple transcripts detected arise by alternative mRNA processing.

The reduction of Ank-1 mRNA levels in *nb/nb* mice with reduced levels of apparently normal-sized Ank-1 and the

presence of a unique (150-kD) form of ankyrin parallel changes described for the murine *spf*^{ash} mutation (Hodges and Rosenberg, 1989). Here, a point mutation produces low levels (10% of wild type) of normal sized preornithine transcarbamylase as well as an aberrant alternatively spliced form of the protein with corresponding low levels of normal length mRNA. Premature termination of translation has been shown to reduce steady-state mRNA levels in human Beta-thalassemia (Baserga and Benz, 1988) and murine muscular dystrophy (gene symbol, *mdx*) (Sicinski et al., 1989). The reduction of mRNA in the presence of a translatable protein, such as occurs in *spf*^{ash} and *nb*, is less well documented. Indeed, Beserga and Benz (1988) showed that no reduction in mRNA levels occurred when translatable missense mutations were made in the human beta globin gene in vitro. However, defective RNA processing, such as occurs in human Beta^{+}-thalassemia (Maquat et al., 1980) can account for decreased mRNA levels (Housman et al., 1973).

In both murine and human Beta-thalassemia, there occur compensatory increases in other types of globins encoded by different genes. For example, fetal gamma (γ) globin is increased in human Beta-thalassemia (Weatherall et al., 1985), while Beta^{minor} globin is increased in murine Beta-thalassemia (Curcio et al., 1987). Hence, the possibility that the 150-kD protein present in *nb/nb* mice is the product of a gene related to, but distinct from, *Ank-1* can not be excluded. The mechanism of mRNA reduction and the origin of the 150-kD protein provide intriguing questions for future studies of the *nb* mutation.

We thank Stan Short and Tammy Packie for expert photographic services, Drs. Edward H. Birkenmeier and Joseph H. Nadeau for helpful comments on the manuscript, and Barbara Dillon for secretarial services.

Received for publication 2 March 1991 and in revised form 27 April 1991.

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