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The murine mutation jaundiced is caused by replacement of an arginine with a stop codon in the mRNA encoding the ninth repeat of β-spectrin

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ABSTRACT **The jaundiced, *ja/ja*, mouse mutant has a severe hemolytic anemia associated with a deficiency of β-spectrin in erythrocyte ghosts. Genes for the disease phenotype and β-spectrin colocalize on Chromosome 12. β-Spectrin mRNA is not detected in reticulocytes or in brain from newborn mutant mice. To locate the nucleotide sequence alteration, the erythroid β-spectrin transcript from mutant spleen was amplified by reverse transcription PCR and sequenced. A C-to-T alteration is present in the mutant transcript and produces a premature stop codon from an arginine codon in mRNA encoding repeat 9 of β-spectrin at amino acid position 1160. The point mutation introduces a *Dde* I site that is present in PCR-amplified DNA of *ja/ja* and *ja/+* mice but not of +/+ control mice from the strain of origin, 129/Sv, or from the two strains, WB/Re and C57BL/6J, in which the mutation has been fixed by over 53 generations of backcrossing. The genetic data confirm that the point mutation is responsible for the severe reductions in β-spectrin mRNA of jaundiced mice.**

The jaundiced mouse mutant exhibits a severe hypochromic anemia uncompensated for by extreme reticulocytosis (1). The homozygous jaundiced (*ja/ja*) mice develop a yellow tint shortly after birth and usually survive only 6–9 days. Newborn mice have greatly enlarged livers and hearts. By 2 days post partum, the jaundiced mice exhibit splenomegaly, while other gross pathological features remain unchanged. Splenectomy does not rescue newborn jaundiced mice, as it does in severely affected humans with recessive hereditary spherocytosis (2), but rather exacerbates the disease (3). This is probably due to the dual role the murine spleen plays in hematopoiesis and in cell destruction.

Hemolytic anemia in both humans and mice is associated with defects in erythrocyte membrane skeleton proteins (4, 5). The major component of the erythrocyte membrane skeleton is spectrin, which forms a two-dimensional meshwork underlying the membrane. Equivalent amounts of related but distinct α- and β-spectrin molecules align in an antiparallel fashion to form heterodimers. Complexity increases by the interaction of heterodimers to form tetramers and higher multimers (6, 7). The spectrin cytoskeleton is not free but attaches to transmembrane proteins in at least two sites. At the tail end of the tetramers, actin and protein 4.1 accumulate, provide linkages between tetramers (8–10), and through interactions of protein 4.1 and the transmembrane protein glycophorin C (11), create a weak linkage to the membrane. At the head end or spectrin heterodimer self-assembly site, β-spectrin is linked to ankyrin (12), which in turn forms a strong attachment to the transmembrane protein, band 3 (13). β-Spectrin appears to be the more important component during nucleation to the erythrocyte membrane (14), although both α- and β-spectrin are essential for stability. Mutations that decrease the cellular complement of α- or β-spectrin or that disrupt dimerization/tetramerization sites usually create fragile erythrocytes with a shortened life span (4, 5).

Initial biochemical analysis of *ja/ja* rbc membrane ghosts revealed an almost complete lack of both α- and β-spectrin as detected by Coomassie blue staining of proteins separated electrophoretically on denaturing gels (15). Antibodies specific for α-spectrin were used to assess the synthesis and translation of α-spectrin in the mutant mice. Nearly normal levels of [^{35}S]methionine-labeled α-spectrin are detected in intact reticulocytes and after *in vitro* translation of mRNA. The newly synthesized α-spectrin is not stably incorporated into the membrane, suggesting the defect is in β-spectrin or ankyrin. Genetic evidence provides proof that *ja* and β-spectrin (gene symbol *Spnb1*) are tightly linked, if not inseparable, on mouse Chromosome 12 (16), whereas ankyrin (gene symbol *Ank1*) maps to Chromosome 8 (17).

Recently, we established the sequence of β-spectrin from normal mice preparatory to initiating a search for the *ja*-mutated site (18). The mouse and human (19) β-spectrin sequences are remarkably conserved. Human β-spectrin is a 220-kDa protein composed of three domains with specific functional roles (20). Domain I consists of the first 273 amino acids, binds actin and band 4.1 (21), and is conserved in the actin-binding region of dystrophin, α-actinin, and slime mold gelation factor (22, 23). The homology with α-actinin continues into the first two repeats of domain II, which contains a total of 17 repeats of usually 106 amino acids with approximately 33% homology between repeating units (19, 20). Each repeat forms three independent α-helices, designated 1, 2, and 3 (20). Adjacent repeats interdigitate through use of helix 3 from the N-terminal repeat with helices 1 and 2 of the next repeat to form a compact helical rod (24). The N-terminal repeats 1–4 include the nucleation site for spectrin dimer formation (25) and the C-terminal region, comprising the 52 amino acids of domain III, are associated with both dimer self-association (26) and phosphorylation (27). The 15th repeat of β-spectrin, near the dimer self-association site, binds ankyrin (12).

In the present paper, we describe use of the normal nucleotide sequence to generate oligonucleotides for the PCR amplification of mutant mRNA. The jaundiced mouse has almost no β-spectrin mRNA in reticulocytes and brain (18). However, the highly erythroid spleen generates low levels of erythroid transcripts and the skeletal muscle produces one novel transcript at apparently normal levels. Given this paradox, it seemed unlikely that the mutation is promoter-related unless some muscle transcripts utilize a unique promoter. Instead, a more logical explanation is that the mRNA is unstable due to presence of a new stop codon or of a splice site alteration. Reverse transcription (RT)-PCR confirmed the former, generated provocative data about exon usage,

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Abbreviation: RT, reverse transcription.

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and provided a molecular mechanism for rapid identification of *ja/ja* and *ja/+* mice.

MATERIALS AND METHODS

RNA and DNA Isolation. Tissues were obtained from 129/Sv (129)-+/+, C57BL/6J (B6)-+/+, WB/Re (WB)-+/+, B6-*ja*/+, WB-*ja*/+, (WB × B6)F_1 (WBB6F1)-+/+, -*ja/ja*, and -sph^{1J}/sph^{1J} mice raised at The Jackson Laboratory. The mice were anesthetized with Avertin and perfused with phosphate-buffered saline (GIBCO/BRL) prior to removal of the tissues for RNA isolation. RNA was isolated by using RNazol B (Cinna/Biotecx, Houston) and was poly(A)-selected with a Stratagene poly(A) Quik mRNA purification kit. For DNA extraction, the mice were killed by cervical dislocation. Liver was removed from the F_1 mice and the spleen was removed from the 129, B6, and WB mice. Liver was homogenized and DNA was extracted by the methods of Miller *et al.* (28). The spleen was homogenized in 2 ml of 20 mM Tris·HCl, pH 7.4/10 mM NaCl/3.0 mM $MgCl_2$. Cell membranes were lysed in 5% sucrose and 4% Nonidet P-40 (Sigma). Nuclei were collected by centrifugation and DNA was prepared by the methods of Kozak and Birkenmeier (29).

RT-PCR and Subcloning. RT-PCR was performed on total RNA from spleens of normal and mutant mice (30). First-strand synthesis used antisense 3′ primers specific for various regions of the normal mouse cDNA sequence (18) as detailed in the text and reverse transcriptase of avian myeloblastosis virus (Promega). After first-strand synthesis, PCR was performed for 40 cycles (each cycle was 94°C for 1 min, 42°C for 2 min, and 72°C for 1 min) on a Perkin–Elmer 9600 thermal cycler. The PCR products were electrophoresed on a 4% NuSieve agarose gel (FMC) and subsequently isolated from low melting temperature agarose (FMC) for ligation into the Invitrogen TA vector and subsequent sequencing.

Nucleotide Sequencing. Sequencing was performed using the dideoxynucleotide chain-termination method according to Sanger *et al.* (31) with Sequenase (United States Biochemical). Data entry and analysis were performed by using Microgenie (Beckman) and PC/GENE (IntelliGenetics) DNA and protein analysis software.

Genomic PCR. PCR was performed on genomic DNA from mutant (homo- and heterozygous) and normal mice by using two primers, designated 64 and 65. The sequences of the primers (5′ to 3′) were TGATCGAAGGCCAGACCGACCCAGATTACC and GATGGCTTCAGCCTGCTTGGCATCTTTCTG. The cycling reaction was 40 cycles (each cycle was 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min). The products were electrophoresed on a 5% NuSieve gel as described in *Results*.

Northern Analysis. Northern analyses were performed by a modification of the method of Sambrook *et al.* (32). RNA was separated by electrophoresis on standard 1% agarose gels containing 1 M formaldehyde in a 1× Mops buffer at 70 V for 6 hr (30). Gels were blotted with 20× SSC (1× SSC = 150 mM NaCl/15 mM sodium citrate, pH 7.0) onto Zetabind (AMF Cuno) and UV-fixed with a Stratalinker (Stratagene). Filters were hybridized with ^{32}P-radiolabeled probes specific for repeats 8–14 of α-spectrin (*Spna1*), for repeats 2–6 and 8–10 of erythroid β-spectrin (*Spnb1*), and for the C terminus of brain β-spectrin (*Spnb2*), where there is the greatest divergence between *Spnb1* and *Spnb2* (33) at 42°C in 5× SSCP (75 mM sodium citrate/600 mM NaCl/75 mM Na_2HPO_4/23 mM NaH_2PO_4)/0.5% sodium dodecyl sulfate (SDS)/40% (vol/vol) formamide/10% dextran sulfate. Filters for Figs. 4 and 5 were washed at 60°C in 0.1× SSC/0.1% SDS (94% stringency) as described previously (18). Filters for Fig. 6 were washed in the same buffer at 50°C (88% stringency) to maximize the intensity of the fainter bands.

RESULTS

Sequence of the Jaundiced cDNA. Determination of the normal mouse β-spectrin cDNA sequence provided the initial impetus and information (18) to sequence the mutant cDNA. RT-PCR was subsequently performed on total spleen RNA from a single surviving *ja/ja* adult mouse. Oligonucleotides for RT-PCR were selected that would generate sequence from nucleotide 799 to nucleotide 393. The RT-PCR products were subcloned in a cloning vector and sequenced. Fig. 1 depicts the nucleotide boundaries of the subclones generated. Each subclone was sequenced, although not always in its entirety. The clone designation and the nucleotides sequenced, as determined by comparison with the normal sequence, are as follows: pJS15, 2673–2908 and 3257–3472; pJS17, 3393–3653 and 3685–3902; pJS19, 4364–4626 and 4615–4767; pJS1, 5991–6255 and 6166–6384; pJS6, 5991–6270 and 6449–6671; and pJS8, 6238–6486 and 6449–6671. The mutation was discovered in subclone pJS17, which contained a C-to-T mutation at position 3621 of the composite nucleotide sequence (Fig. 2). In fact, the amino acid sequence is otherwise remarkably conserved between the B6-+/+ β-spectrin (18) and the mutant that was derived originally from the 129 strain. The C-to-T alteration produces a translational stop codon from an arginine codon at position 1160 of the deduced amino acid sequence in repeat 9.

Confirmation of the Mutation. The mutation introduces a *Dde* I site and allows for detection of the mutation without sequencing the PCR products. To confirm that the site is present in *ja/ja* mice other than the surviving adult tested originally but is not represented in control mice, RT-PCR amplification of nucleotides 3393–3902 was performed. Splenic mRNA was extracted from a *ja/ja* neonate and from an sph^{1J}/sph^{1J} adult, a mutant with hemolytic anemia caused by an anomaly in α-spectrin localized to Chromosome 1 (15, 16), and the RT-PCR products were digested with *Dde* I. The jaundiced product but not the product from the mice with spherocytosis is cut with the enzyme (data not shown).

To confirm that the nucleotide alteration is the mutation, the genesis and subsequent breeding history of the mutation were exploited. The mutation arose spontaneously in 1959 on the 129/Sv inbred mouse strain (34). In 1961, after 13 generations of brother–sister mating, the mutation was moved onto both the B6 and WB inbred mouse strains by continuous backcrossing; it has been maintained by forced heterozygosis ever since. Currently, the mutation is 53 generations removed from the stock of origin. Thus, one would expect to detect the *Dde* I site in PCR-amplified genomic DNA of WBB6F1-*ja/ja*, WB-*ja*/+, and B6-*ja*/+ mice but not of WBB6F1-+/+, WB-+/+, B6-+/+, or 129-+/+ mice. A

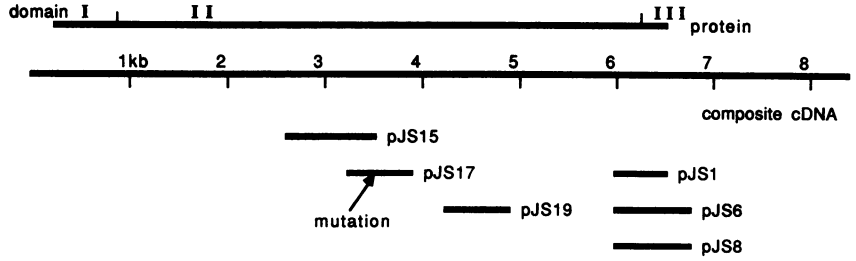

FIG. 1. Subclones of RT-PCR products for an adult *ja/ja* mouse. The subclones (pJS) are noted below a scale for the DNA length in kb. A linear schematic of the protein structure is present above the DNA scale. I, II, and III correspond to the β-spectrin domains. The mutation is noted by an arrow.

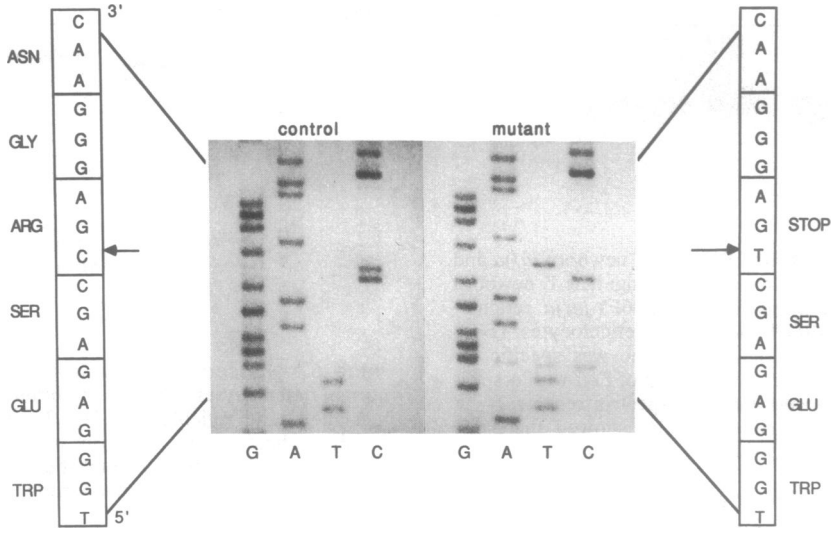

FIG. 2. Partial sequence of the RT-PCR subclone from control (sph^{1J}/sph^{1J}) and mutant (ja/ja) spleens. The β-spectrin transcripts from mutant and normal mice were amplified from nucleotides 3393–3902 (18). The amplification products were subcloned and sequenced. The autoradiograph of the sequencing gel is shown in the center for nucleotides 3613–3630. The corresponding nucleotide sequences are noted to the left and right for the control and mutant, respectively. The arrows denote the position of the mutation.

170-bp genomic DNA segment from nucleotide 3523 to nucleotide 3692 that encompasses the mutation was amplified and the product was digested with *Dde* I (Fig. 3). The amplified product was of the expected size, indicating that there are no introns present in this particular genomic sequence and discounting the hypothesis that the transition and decreased message levels are caused by a defect in a primary splice site. The 129-+/+ DNA is not digested, suggesting that the sequence alteration is not a polymorphism from the strain of origin. The other normal controls (WB, B6, and WBB6F1) also do not cut, indicating that the normal alleles from these mice do not contribute to the 100- and 70-bp *Dde* I digestion products. The fixation of the mutation in both genetic backgrounds is demonstrated by the fact that heterozygous mice from both the WB and the B6 backgrounds show both full-length and digested PCR products. Only digested fragments are present in the PCR product of ja/ja DNA.

Northern Analysis of Mutant Transcripts. Previously, we showed that the numerous β-spectrin transcripts in reticulocytes and brain may utilize distinct exons that have not yet been identified (18). For example, a 7.2-kb normal adult muscle transcript contains repeats 2–6 sequence but, unlike the 11- and 10.3-kb muscle transcript, does not include domain I or repeats 16 and 17. This raises the question as to the sequence of the 7.2-kb transcript and how it differs from the 11- and 10.3-kb muscle β-spectrin transcripts that are more typical of reticulocytes. A question posed by the current experiments is whether the exon encoding the mutated region is represented in the transcripts that appear to be missing in jaundiced mice. Consequently, the transcript pattern in the normal and jaundiced mice was reinvestigated using region specific probes, one of which includes repeat 9, the site of the mutation. β-Spectrin transcripts of 8.0 and 9.6 kb are detected by Northern analysis of reticulocyte RNA from newborn B6-+/+ and from adult WBB6F1-sph^{1J}/sph^{1J} controls but not from newborn WBB6F1-ja/ja mice when a probe specific for β-spectrin repeats 2–6 is used (Fig. 4). One might expect that since these transcripts are missing in the jaundiced mice, they would include repeat 9. As expected, the same pattern of hybridization is detected by using a probe to β-spectrin repeats 8–10 (data not shown). Reprobing of the stripped Northern blot with an α-spectrin probe for repeats 14–18 (35, 37) shows α-spectrin transcripts in the ja/ja and control lanes, indicating RNA is present in each lane, although the concentration in the ja/ja lane appears less, possibly a secondary consequence of the β-spectrin defect.

A developmentally regulated 10-kb β-spectrin transcript is detected in the newborn B6-+/+ brain but not in the brain of newborn jaundiced mice when a probe for β-spectrin repeats 8–10 is used (Fig. 5). The various controls show appropriate patterns. The control 8.0- and 9.6-kb transcripts are present in the sph^{1J}/sph^{1J} adult reticulocytes. Reprobing the stripped Northern blot with a probe for brain β-spectrin (*Spnb2*) (38), which maps to Chromosome 11 (39), shows equivalent transcript levels in the brain of ja/ja and +/+ and, as expected, no message in the reticulocytes. Conversely, an erythroid α-spectrin probe specific for repeats 14–18 shows mRNA

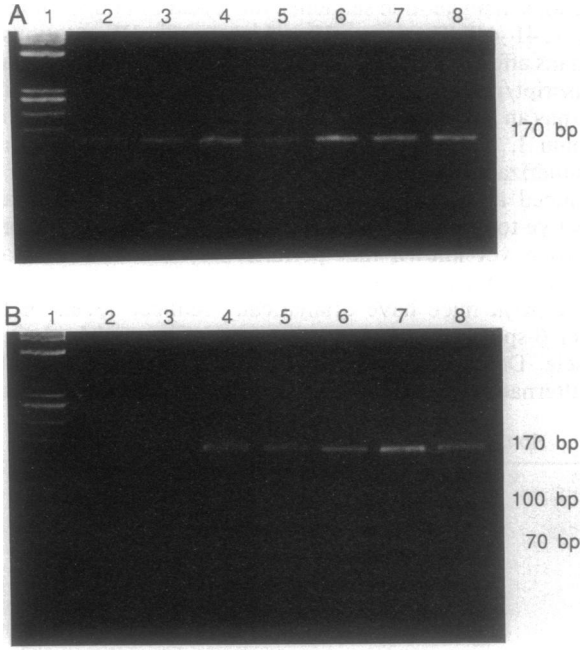

FIG. 3. Restriction digest of genomic PCR products from normal and mutant mice. (*A*) Undigested PCR product. (*B*) PCR products digested with *Dde* I. The DNA samples used for PCR analysis are as follows: lane 1, marker DNA, *Hae* III fragments of φX174 DNA; lane 2, 129-+/+; lane 3, WBB6F1-ja/ja; lane 4, WBB6F1-+/+; lane 5, WB-+/+; lane 6, WB-ja/+; lane 7, B6-+/+; and lane 8, B6-ja/+.

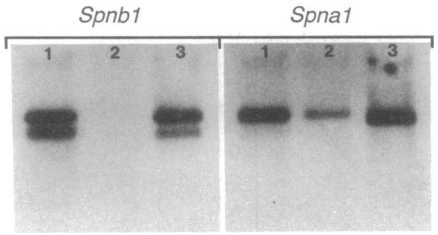

FIG. 4. Northern analysis of total RNA from newborn *ja/ja* and normal reticulocytes. RNA was from the following: lane 1, newborn B6-+/+ reticulocytes; lane 2, newborn WBB6F1-*ja/ja* reticulocytes; and lane 3, adult WBB6F1-sph^{1J}/sph^{1J} reticulocytes. (*Left*) The probe used was for β-spectrin (*Spnb1*) and was specific for β-spectrin repeats 2–6 (18). (*Right*) Rescreening of *Left* with α-spectrin (*Spna1*) pB129, which is specific for α-spectrin repeats 14–18 (35). The *Spnb1* probe for repeats 8–10 (36) showed the same hybridization pattern as that for β-spectrin repeats 2–6.

only in the sph^{1J}/sph^{1J} reticulocytes and not in newborn brain. Thus, it appears the deficiency of the erythroid β-spectrin in both brain and reticulocytes of the *ja/ja* mice is linked to transcripts that contain the mutated region.

Further proof is shown in Fig. 6. A Northern blot shown previously (18) to contain the normal adult β-spectrin transcripts—reticulocyte 9.6 and 8.0 kb; muscle 11.0, 10.3, 7.2, and 4.0 kb; and brain 11.0 kb—was hybridized with the repeat 8–10 probe. All except muscle 7.2- and 4.0-kb transcripts contain repeats 8–10. The 7.2-kb transcript is present at normal levels in *ja/ja* skeletal muscle. The concentration of the 4.0-kb transcript has not yet been compared in mutant and normal muscle.

DISCUSSION

The characterization of the mutation in jaundiced mice is the major finding of this report. Interestingly, the mutation is in an exon that encodes repeat 9 of the β spectrin. This is an unusual site, since all but one (40) of the β-spectrin mutations identified to date in humans involve point mutations and/or deletions in nucleotide sequence for repeat 17 and/or domain III (24, 41–44). The repeat 17 and/or domain III mutations in humans affect spectrin tetramerization and, for the most part, transcript/protein levels are unaffected. β-Spectrin Kissimmee has an Arg instead of a Trp at codon 202 in the N-terminal domain I, a change that affects protein 4.1 binding, not tetramerization (40). β-Spectrin Kissimmee appears to be produced at normal levels, but the protein is abnormally sensitive to oxidation (45). Thus, *ja* in the mouse is the only mutation yet known that permits assessment of message stability and message usage in various tissues.

The *ja/ja* mice have dramatically reduced levels of the larger β-spectrin mRNA species in reticulocytes, brain, and muscle. Defects in mRNA synthesis, processing, stability, or, alternatively, in cell maturation are possible causes of the

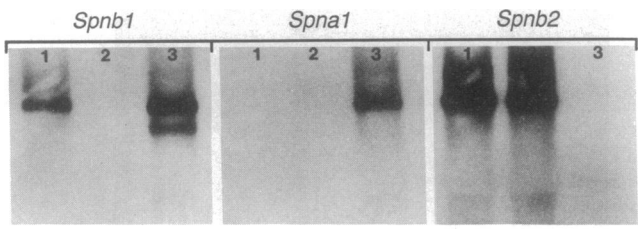

FIG. 5. Northern analysis of total RNA from brain and reticulocytes. RNA was from the following: lane 1, newborn B6-+/+ brain; lane 2, newborn WBB6F1-*ja/ja* brain; lane 3, control adult WBB6F1-sph^{1J}/sph^{1J} reticulocytes. Probes are *Spnb1* erythroid β-spectrin repeats 8–10 (36); *Spna1* erythroid α-spectrin repeats 14–18 (35); and *Spnb2* unique C terminus of brain β-spectrin (38, 39).

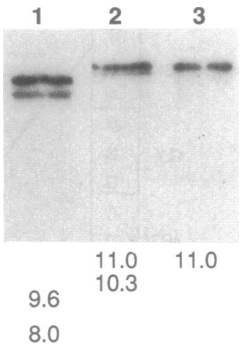

FIG. 6. Northern analysis of poly(A)-selected RNA (1.0 μg) from normal reticulocytes, muscle, and brain. RNA was from the following: lane 1, adult reticulocytes; lane 2, adult skeletal muscle; and lane 3, adult whole brain. The probe was *Spnb1* erythroid β-spectrin repeats 8–10 (36). Previous published results (18) detected a strong signal for a 7.2- and a 4.0-kb transcript in lane 2 on this Northern blot.

phenotype. It is unlikely that the reduced steady-state transcript levels of β-spectrin mRNA in the jaundiced mice are due to an mRNA processing defect caused by the mutation. We have shown that the segment amplified from the genomic DNA is the expected length (170 bp), indicating that no introns are present. The genomic PCR product appears to be part of an exon whose boundaries are not yet characterized for β-spectrin. It is, of course, possible that the mutation creates a cryptic acceptor splice site but highly unlikely that it destroys an existing one. The sequence is altered by the mutation from GAGAGCCGAG to GAGAG↓CTGAG, with the putative acceptor splice site noted by the arrow. A similar splice site exists in exon 42 (repeat 19) of human α-spectrin (46). If this is a cryptic splice site, one would expect the resultant transcript to be shorter and the coding sequence to be out of frame until after the next intron–exon boundary. We have no evidence for the existence of such a transcript. We have been unable to obtain an RT-PCR product from mutant reticulocytes, but the spleen RT-PCR product shows a sequence alteration only at the site of the mutation. Interestingly, the Arg mutated at residue 1160 is one of the consensus amino acids conserved in all repeats of both α- and β-spectrin, so any protein products involving readthrough of the stop codon might have limited function.

It is not unusual for nonsense mutations to be associated with reduction of steady-state message levels. For example, the mouse model for human mucopolysaccharidosis type VII (MPS VII) contains no detectable β-glucuronidase activity and dramatically reduced mRNA levels (47). A single base pair deletion leads to the introduction of a premature stop codon in β-glucuronidase of MPS VII mice (48). Nonsense mutations affect steady-state transcript levels *in vivo* in dihydrofolate reductase mutant Chinese hamster ovary cells (49). Additionally, the presence of a premature translation termination codon in the β-globin gene affects the accumulation of β-globin mRNA (50). On the basis of this discussion, we predict that the *ja/+* mice might have half-normal levels of β spectrin transcripts, a prediction that could be tested with allele-specific probes.

It is interesting that neither exon skipping nor insertion of premature stop codons at the 3′ end of human β-spectrin appears to dramatically alter transcript levels, although the protein generated can be unstable (51). It is important to remember, however, that the human patients, unlike the *ja/ja* mice, are heterozygous. Therefore, half-normal levels of β-spectrin mRNA are expected, and the patients are generally less severely affected than the mice. It is possible that the location of the exons and stop codons may influence the mRNA disposition. In the human patients, exon skipping or

premature stop codons have thus far been seen only in the 3′ translated end, whereas the mouse mutation is in the middle of the transcript. As expected, the proteins generated from the human transcripts are smaller. No erythroid β-spectrin has been detected in *ja/ja* reticulocytes (15), although it may be present in striated muscle.

Northern comparisons of equivalent amounts of total RNA show no β-spectrin and decreased levels of α-spectrin in the *ja/ja* vs the +/+ blood cells (Fig. 4), indicating that the mutant cells are generating less spectrin message. While it is possible that diminution of β-spectrin mRNA may directly affect α-spectrin mRNA levels, we have no evidence for such a phenomenon. Instead, we have shown during *in vitro* translation that there are higher levels of *ja/ja* than of +/+ α-spectrin synthesized from comparable amounts of mRNA (15). It seems highly unlikely that mutant α-spectrin translation is more efficient *in vitro*. The translation data suggest that two alternative explanations of the decreased α-spectrin RNA levels noted in the current experiment—i.e., differences in the relative reticulocyte age or in mRNA extractability—are not likely. Therefore, it appears the decreased mutant α-spectrin may be an aberration.

Previously, we demonstrated the complexity of transcripts in the normal mouse and suggested there were as yet unidentified coding regions (18). In the present paper, we have shown that the β spectrin transcripts missing in the jaundiced reticulocytes and brain have the mutated ninth repeat, but the 4.0- and 7.2-kb transcripts in muscle skip the exon encoding this region. The fate of the 4.0-kb transcript is unknown, but the 7.2-kb transcript is present at normal concentration in the skeletal muscle of the jaundiced mouse (18). Interestingly, the level of a 7.2-kb mRNA is decreased in cardiac muscle of newborn *ja/ja* mice. Use of the repeat 8–10 probe should provide interesting insights into the differences between the 7.2-kb transcripts in skeletal and cardiac muscle. Concurrent pathological examination will detect any subtle differences in skeletal and cardiac muscle that may be related to transcript deficiency. This could prove relevant to humans, where alternative 3′ ends are used in muscle and reticulocytes. It is unclear currently whether the many human mutations affecting the 3′ end of the β-spectrin impinge on the muscle transcript levels or histopathology.

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