Hereditary Overexpression of Adenosine Deaminase in Erythrocytes: Studies in Erythroid Cell Lines and Transgenic Mice

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Overexpression of adenosine deaminase (ADA) in red blood cells is characterized by a marked, tissue-specific increase in levels of structurally normal ADA mRNA and enzymatic activity in the erythrocytes of affected individuals, leading to adenosine triphosphate (ATP) depletion and hemolytic anemia. This autosomal dominant trait is linked to the ADA gene. To investigate the molecular mechanism responsible for this disorder, we examined relative reporter gene activity using constructs containing 10.6 kb of 5' flanking sequence and 12.3 kb of the first intron of the ADA gene from the normal and mutant alleles. No differences in chloramphenicol acetyltransferase (CAT) activity were found in transient transfection experiments using erythrocyte cell lines.

Adenosine deaminase (ADA) is a purine catabolic enzyme that catalyzes the deamination of adenosine to inosine and 2'-deoxyadenosine to 2'-deoxynosine. The ADA gene is expressed in all tissues, and thus may be categorized as a "housekeeping" gene. However, the level of expression varies by more than 1,000-fold in different tissues and developmental states, with the highest level of expression in cortical thymocytes and in T lymphoblasts. This high level of expression in immature T cells may be critical for T-cell development, because mutations in the ADA gene that abolish its function cause severe combined immunodeficiency disease.

In contrast to immature T cells, red blood cells (RBCs) normally have low amounts of ADA activity. Mutations in the ADA gene that result in decreased or absent activity have no effect on the function or longevity of the RBC; however, the tissue-specific overproduction of ADA in RBCs causes hemolytic anemia. Individuals with this autosomal dominant disorder have shortened 51Cr-labeled RBC survival, elevated reticulocyte count, splenomegaly, and mild hyperbilirubinemia. RBCs from affected individuals have 40- to 70-fold increased levels of ADA activity, leading to the increased catabolism of adenosine and adenosine triphosphate (ATP) depletion, while ADA activities in leukocytes and fibroblasts are normal. The specific molecular defect underlying the tissue-specific enzyme overexpression has not been elucidated.

Since the disorder is associated with a 70-fold increase in the amount of catalytically normal ADA protein and a marked increase in the level of structurally normal ADA mRNA, it seems likely that the defect is due to increased production in erythroid precursors. From linkage analysis, we have determined that the mutation causing RBC-specific ADA overproduction lies within or near the ADA locus. This mutation most likely exists in a region of the gene that affects either transcription or pre-mRNA processing, since the mRNA is normal in sequence. In order to search for the mutation in potential regulatory regions 5' to and within the first intron of the 32-kb ADA gene, we generated reporter gene constructs from the normal and putatively aberrant ADA alleles and looked for differences in expression in erythroid cells using transient transfection assays and in a transgenic mouse model.

MATERIALS AND METHODS

Cell culture. K562 (obtained from Dr Francis Collins, NIH) and K562-BM7 (obtained from Dr George Atweh, SUNY-Brooklyn) erythroleukemia cells were maintained in RPMI-1640 with 10% fetal calf serum. Mouse erythroleukemia (MEL) cells obtained from Dr Mike Clarke (University of Michigan) were grown in Dulbecco's modified essential medium (DMEM) with 10% fetal calf serum. Hemoglobin synthesis in K562 cells was induced by treatment with 20 µmol/L hemin for 4 days.

DNaSe I hypersensitive sites. Hypersensitive sites were determined by an adaptation of a previously described method. 106 cells were washed with cold phosphate-buffered saline, resuspension buffer (RSB) (10 mmol/L Tris, pH 7.4, 10 mmol/L NaCl, 5 mmol/L MgCl2), and RSB + 0.5% NP-40. Nuclei were resuspended in 4.5 mL RSB + 1 mmol/L CaCl2, divided into 0.5 mL aliquots, and digested with DNase I (Worthington, Freehold, NJ) at a final concentration of 0 to 2 µg/mL at 37°C for 15 minutes. The reactions were stopped with 600 mmol/L NaCl, 20 mmol/L Tris, pH 7.4, 5 mmol/L EDTA, 1% sodium dodecyl sulfate (SDS). DNA was purified by proteinase K treatment, phenol/chloroform extraction, and ethanol precipitation. DNase-digested DNA was cut with HindIII or BamHI, electrophoresed on a 1% agarose gel, and blotted to nitrocellulose. Probes were generated by polymerase chain reaction (PCR) amplification of ADA gene sequences and labeled as described.

Library construction and cloning. Genomic DNA from an affected individual (E.L.) was prepared from Epstein-Barr virus-transformed B lymphoblasts and digested to completion with BamH1. DNA migrating between 9 and 23 kb on an agarose gel was excised, purified with GeneClean (Bio 101, La Jolla, CA), and ligated into the Lambda Fix II vector (Stratagene, La Jolla, CA) at the Xho1 site.
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site by partial fill-in using a 3:1 vector:insert ratio. DNA was packaged using Gigapack II Gold extract (Stratagene), and Tap 90 cells were infected. Plaques, 5.4 × 10⁶, were screened for the 12.6-kb promoter and 12.3-kb intron fragments. Eight promoter clones and 9 intron clones were plaque-purified. Phage DNA was prepared by the plate lysis method with the addition of a second polyethylene glycol precipitation.

Promoter chloramphenicol acetyltransferase (CAT) constructs were made by ligating the 2.2-kb NcoI genomic fragments containing 2.1 kb of promoter and 98 bp of 5' untranslated sequence into the pCAT-Basic vector (Promega, Madison, WI) at the XhoI site to form pADA CAT 2.2. The 8 clones were sequenced using a primer to an AluVpA11 element within the insert to distinguish the two alleles. To obtain additional 5' sequences, the 10.5-kb EagI-SalI fragments from the BAC genomic clones were ligated into pADA CAT 2.2 digested with EagI and SalI to generate pADA CAT 11.

The 12.3-kb BamHI intron fragments were subcloned into pADA CAT 11 at the BamHI site 3' to the CAT gene. pADA CAT 11 (mutant and wild-type) vectors were digested with BamHI and partially filled in with dGTP and dATP, whereas the intron inserts were digested with SalI and partially filled in with dCTP and dTTP. Ligation yielded pADA CAT 11/12.

Transient transfection assays. K562, K562-BM, and MEL cells were grown to log phase, washed with serum-free medium, and resuspended in serum-free medium at 10⁶ cells/400 μL. Plasmid DNA was prepared with Qiagen columns (Chatsworth, CA), followed by centrifugation to remove resin and phenol/chloroform extractions of the supernatant. Forty micrograms of DNA in 100 μL of phosphate-buffered saline was added to the cells in constant molar amounts. Electroporation was performed using a Bio-rad Gene Pulser (Richmond, CA) and 0.4 cm cuvettes; settings were 240 V, 960 μF, and partially filled in with dCTP and dTTP. Where the intron inserts were digested with SalI and partially filled in with dCTP and dTTP. Ligation yielded pADA CAT 11/12.

Transgenic mouse assays. The ~25-kb ADA CAT 11/12 inserts were liberated from the plasmid vector by digestion with SalI and PvuII, separated by agarose gel electrophoresis using Seaplaque-GTG (FMC, Rockland, ME), extracted with phenol, and precipitated with ethanol. The DNA was further purified using a Qiaquick TIP-20 column and dissolved in 10 mmol/L Tris, pH 7.4, 0.05 mmol/L EDTA. Transgenic mice were prepared by the Transgenic Animal Model Core of the University of Michigan’s Biomedical Research Core facilities. The purified DNA was microinjected into F1 hybrid zygotes from C57BL/6J × SJL/J parents at a concentration of 2 to 3 ng/μL. After overnight incubation, the eggs that survived to the 2-cell stage were transferred to day 0.5 postcoitum pseudopregnant CD-1 females. C57BL/6J × SJL/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME), and CD-1 mice were obtained from Charles River (Wilmington, MA). Founder transgenic mice were mated to C57BL/6J mice. All procedures using mice were approved by the University of Michigan Committee on Use and Care of Animals and the University of North Carolina Institutional Animal Care and Use Committee. All work was conducted in accord with the principles and procedures outlined in the NIH Guidelines for the Care and Use of Experimental Animals.

To assay for the presence of the transgene, mouse tail DNA was prepared and amplified by PCR using primers specific for the human ADA gene and primers specific for the mouse β-globin gene as an internal control. Southern blot analysis was used to determine the different lineages in the F1 generation. In addition to the bands expected from the usual head-to-tail integration pattern, other bands were observed that most likely represented partial digestion products, rearrangements, or deletions. To get an approximate estimate of copy number, we performed slot blot assays using serial dilutions of pADA CAT 11/12 in 10 μg of mouse genomic DNA as standards. Quantitation was performed with a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Preparation of adult mouse tissue extracts and CAT assays were performed as described by Aronow et al. Blood was collected by retroorbital bleeding or cardiac puncture, and RBCs were purified on Histopaque 1083 (Sigma, St Louis, MO). Solid tissues were homogenized in 300 μL 0.25 mol/L Tris, pH 8, with a Polytron homogenizer. All tissues were subjected to three freeze-thaw cycles and centrifugation. Protein concentrations of the supernatants were determined by the method of Bradford using bovine serum albumin as a standard. CAT assays were performed on all tissues from a single mouse on the same day. Twenty microliters of extract was heated to 63°C for 10 minutes before addition of acetyl CoA (25 μg) and [14C]chloramphenicol (0.05 μCi) for a 2-hour incubation. Extracts were diluted in 10 ng/mL bovine serum albumin if necessary to ensure linear assay conditions. Acetylated products were separated by thin layer chromatography and visualized by autoradiography. Quantitation was performed by counting the monocacylated product spots in a liquid scintillation counter.

**RESULTS**

To determine potential endothelial-specific regulatory sites for ADA expression, the ADA gene in K562 erythroblastemia cells was examined for DNase I hypersensitive sites. Although this assay was of necessity, performed in a leukemic cell line and not in cells from an affected individual, we hypothesized that the resulting hypersensitive sites might still be informative. Figure 1 shows the 5' and first intron

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Fig 1. Schema of the 5' and first intron regions of the ADA gene examined for DNase I hypersensitive sites. BamHI and HindIII sites and major DNase I hypersensitive sites (HS) are indicated. Probes 1 and 2 were used to scan the 5' flanking region, first exon, and part of the first intron. Probes 3 and 4 were used to scan much of the first intron.
regions scanned with this technique. The HindIII digest was first hybridized with probe 1 to examine the 7.6-kb region surrounding the first exon (Fig 2A). A strong hypersensitive site, indicated by the arrow, was found at the exon 1-intron 1 junction (see also HS in Fig 1), and several minor sites were observed further 5'. Hemin, a weak inducer of hemoglobin synthesis and hence of erythroid differentiation, had no detectable effect on DNase hypersensitivity, but induced benzidine positivity in only 30% of treated cells. The hypersensitive sites were confirmed by scanning the 12.6-kb BamHI fragment containing 10.5 kb of upstream sequence, exon 1 and 2 kb of intron with a second probe from the opposite side (data not shown).

BamHI digestion also released the adjacent 12.3-kb fragment encompassing most of the first intron (Fig 1). There was a strong hypersensitive site 8 to 9 kb into the first intron (Fig 2B) that closely mapped to a previously reported hypersensitive site1 (see also HS V in Fig 1). Interestingly, hemin treatment decreased the intensity of this site, suggesting that erythroid differentiation might decrease protein binding to this site. This hypersensitive site was confirmed by digestion of the DNase-treated DNA with HindIII and labeling with a different probe (data not shown).

Genomic DNA from immortalized B lymphoblasts from an affected individual was digested to completion with BamHI and used to prepare a library containing 9- to 23-kb fragments. Eight promoter clones were isolated, and the mutant and wild-type alleles were identified by sequencing through an AluVpA11 region. Nine intron clones were isolated and sequenced in the region mapping to hypersensitive site V. Six of 9 clones had an A to C transversion when compared with the published sequence,5 creating a new BstNI site. To determine whether this base change was linked to the mutant allele, we traced the inheritance of the BstNI restriction fragment length polymorphism (RFLP). PCR of hypersensitive site V was done on DNA samples from 7 members of the immediate family, and the BstNI RFLP was found to be associated with the unaffected allele. Thus, the A to C transversion at hypersensitive site V of the first intron was a neutral polymorphism in the wild-type allele.

The separation and identification of the two alleles allowed us to compare their expression using transient transfection assays. Three pairs of plasmids were prepared: pADA CAT 2.2, containing 2.1 kb of promoter and 98 bp of 5' untranslated sequence; pADA CAT 11, containing 10.6 kb of upstream sequence; and pADA CAT 11/12, containing both 10.6 kb of upstream sequence and 12.3 kb of intron 1 (Fig 3). Three cell lines were tested: K562 erythroleukemia cells, which express small amounts of embryonic and fetal hemoglobins; K562-BM cells, which express a small amount of adult β-globin as well; and MEL cells, which express some adult mouse globin. As seen in Fig 3, no significant differences in CAT activities were observed between the two alleles, regardless of construct or cell type.

Since the erythroleukemia cell lines do not recapitulate normal erythroid differentiation, it remained possible that the mutant allele might be overexpressed as a consequence of its failure to undergo normal downregulation during the maturation of normal erythroid progenitors. In addition, it seemed possible that integration of the construct into the genome might be required for its regulation. We therefore made transgenic mice containing either mutant or wild-type ADA CAT 11/12. All founder mice were bred, and only F1 mice were analyzed to avoid the problem of mosaicism.
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Twelve lines of mice with the mutant allelic construct and 7 lines with the wild-type allelic construct were obtained. All mice with either the mutant or wild-type transgene expressed CAT activity. Those mice with low copy numbers of the transgene (generally <10 copies) expressed CAT in the thymus, spleen, and marrow in a consistent fashion, with expression in the thymus being greater by at least two orders of magnitude (Fig 4A). Expression in the brain, liver, and kidney was not always detectable, and no expression was detected in RBCs with either allele. Treatment of mice with phenylhydrazine to induce hemolysis and reticulocytosis did not induce CAT activity in RBCs (data not shown).

In order to obtain detectable RBC CAT expression, we examined the mice with transgenes in high copy numbers (Fig 4B). Seven lines had the mutant allelic transgene in high copy numbers, and 3 lines had the wild-type allele in high copy numbers. Again, thymus had the highest level of expression; marrow and spleen had 100-fold less. Expression in the brain, liver, and kidney was lower than in the thymus by 1,000- to 10,000-fold. CAT activities in RBCs were low, but easily detectable in these mice. There was no consistent difference in the level of expression between mutant and wild-type allelic constructs in RBCs. Table 1 shows RBC CAT activity normalized to CAT activity in the thymus or kidney. In 6 lines with the mutant allelic construct, normalized RBC CAT activities were not significantly greater than in the 3 lines with the wild-type allelic construct. Only line 9784b had significantly elevated RBC CAT activity, a finding which may be attributed to its integration site in the genome.

**DISCUSSION**

Elevated adenosine deaminase levels in RBCs have been reported in numerous hematological disorders, such as Diamond-Blackfan syndrome, AIDS, Diamond-Blackfan syndrome, and primary acquired sideroblastic anemia. ADA activity in these disorders is usually elevated by 2- to 10-fold and is most likely secondary to defective erythropoiesis. When ADA activity is 40- to 110-fold above normal, it is the primary defect and causes hemolytic anemia by depleting the RBC of ATP. Four cases of ADA overproduction associated with hemolytic anemia have been reported, but only one kindred has been demonstrated to have an autosomal dominant mode of inheritance with the genetic defect linked to the ADA locus. This association made it feasible to attempt to identify the precise molecular defect in this disorder.

In earlier studies, we had determined that increased RBC ADA activity was due to an increased level of ADA mRNA. A cDNA clone from a reticulocyte library of an affected individual was completely normal in sequence. This result was confirmed in other family members as well by chemical mismatch cleavage analysis (E. Chen, unpublished data). Furthermore, in six other clones from the reticulocyte library, the 5' and 3' untranslated regions were examined for base alterations. None were found, suggesting that cytoplasmic ADA mRNA was unlikely to be more stable. When the 1.1-kb XhoI fragment encompassing the proximal promoter region of the ADA gene was cloned from an affected individual, no sequence alterations were found in the 8 clones examined (E. Chottiner, unpublished data). From these findings, it was clear that the mutation was very unlikely to reside in the coding region or in the promoter of the gene. We therefore proceeded to look for sites of ADA gene regulation both in more distal 5' regions and within the first intron. This intron contains a classical enhancer that boosts expression in T-lymphoblast cell lines, as well as a locus control region (LCR) that confers copy number-dependent, position-independent expression of a transgene in the thymus. Other regions of the ADA gene, including the other introns and the 3' flanking sequences, may also contain regulatory regions, but none have been described thus far.

To locate possible erythroid-specific regulatory regions,
the ADA gene was scanned for DNase I hypersensitive sites from 10.6 kb upstream of the translation start site to 14.3 kb into the first intron. Two major hypersensitive sites were found in K562 erythroleukemia cells. The one at the first exon was hemin-insensitive and corresponds to the region reported to be involved in transcriptional attenuation27,28 (discussed below). The other major site was in the middle of the first intron, and corresponds to hypersensitive site V, as
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Table 1. Ratio of Transgenic Mouse RBC CAT Activity to Thymus or Kidney CAT Activity

<table>
<thead>
<tr>
<th>Mutant lines</th>
<th>RBC/Thymus (×10^6)</th>
<th>RBC/Kidney (×10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9974 a</td>
<td>3.3</td>
<td>0.03</td>
</tr>
<tr>
<td>9774 b</td>
<td>2.5</td>
<td>1.0</td>
</tr>
<tr>
<td>9774 a'</td>
<td>10.5</td>
<td>0.69</td>
</tr>
<tr>
<td>9774 d</td>
<td>6.2</td>
<td>2.5</td>
</tr>
<tr>
<td>9784 b</td>
<td>500</td>
<td>19</td>
</tr>
<tr>
<td>9782 a</td>
<td>4.2</td>
<td>0.84</td>
</tr>
<tr>
<td>9773</td>
<td>6.6</td>
<td>0.28</td>
</tr>
<tr>
<td>Wild-type lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8951 a</td>
<td>4.7</td>
<td>1.9</td>
</tr>
<tr>
<td>8951 b</td>
<td>1.4</td>
<td>1.1</td>
</tr>
<tr>
<td>8951 c</td>
<td>1.1</td>
<td>0.70</td>
</tr>
</tbody>
</table>

described by Aronow et al.1 This site is found in many tissues and cell lines, including human thymus, transgenic thymus, transgenic spleen, Molt-4 T lymphoblasts, and CEM T lymphoblasts, as a minor hypersensitive site. Hypersensitive site V is very strong in fibroblasts, but is not seen in the B-cell line GM 3638, and is distinct from the site responsible for the very high levels of thymic expression. Whether or not these hypersensitive sites exist in the erythroid progenitors of affected individuals or constitute important sites for ADA down-regulation, which, when disrupted, would increase ADA expression, could not be determined from these studies.

To determine the physiologic significance of these regions, we examined the relative CAT expression of constructs containing the mutant and wild-type alleles. In our transient transfection assays, using three cell lines that represent to some extent different developmental stages of erythroid cells, we saw no difference in expression between the two alleles. Transient transfection into erythroid cell lines has some drawbacks, which have been similarly encountered by those studying the non-deletion form of hereditary persistence of fetal hemoglobin (HPFH). Various possibilities could explain both our results and those in the HPFH experiments. First, and we believe most likely, the causal mutation is not in our transgene. It could be further 5' to the 10.6-kb fragment, in other introns, or at the 3' end. Second, the transgene could contain the causal mutation, but the mutation may not be expressed outside of its native genomic context. For example, the causal mutation may have to interact with normal LCR elements that are not present in our constructs, but are present in more distant regions of chromosome 20. Other possibilities exist, but are far less likely. For example, failure to detect overexpression could be due to the absence in the construct of the last 31 bp of exon 1 and the adjoining 2 kb of first intron. However, we have not found any sequence differences between the two alleles within the initial 594 bases of the first intron, the region that has been implicated in transcriptional attenuation of ADA gene expression.27,28 The demonstration of correct tissue-specific expression of our transgene in the absence of this region makes its functional role uncertain. Indeed, there is increasing evidence that transcriptional attenuation of mammalian genes may be an artifact of the Xenopus oocyte system used to study this phenomenon.25,26 It is also conceivable that the appropriate trans-acting factor(s) involved in RBC ADA overexpression are not present in murine erythroid precursors. However, the mouse model has served very well for studies of globin gene regulation and we are not aware of any examples of erythroid-specific transcription factor discrepancies between mice and humans.

Further extension of this work would require the cloning of the entire mutant and wild-type ADA genes, including large 5' and 3' flanking regions, in yeast artificial chromosomes or bacterial artificial chromosomes.37 Until better systems develop for studying human erythropoiesis, introduction of this large transgene into the mouse remains the most direct approach. The feasibility of introducing large transgenes has been demonstrated recently.39-41 Elucidating the mechanism of ADA overexpression in RBCs could provide new insights into the regulation of gene expression during erythroid development.
ACKNOWLEDGMENT

We thank David Ginsburg for his continuous encouragement and advice. We also thank Craig Thompson for the DNase hypersensitive site assay. Deborah Gumucio for the K562 transient transfection assay, Sally Camper and Thom Saunders for generation of transgenic mice, Bruce Aronow and Dan Wiginton for sharing their experience and technical knowledge, and Elaine Chottiner and Tom Gribbin for their initial efforts on this project.

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