Erythrocyte Adenosine Deaminase Overproduction in Hereditary Hemolytic Anemia

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A marked tissue-specific increase in erythrocyte adenosine deaminase (ADA) activity is associated with an autosomal dominantly inherited hemolytic anemia. We investigated the molecular basis of ADA overproduction by studying reticulocyte ADA mRNA from affected individuals. Analysis of proband reticulocyte ADA cDNA clones revealed normal sequence. RNase mapping demonstrated that the amount of ADA mRNA in affected reticulocytes was greater than the amount in normal B lymphoblasts, whereas ADA mRNA was undetectable in normal reticulocytes. The 5'- and 3'-untranslated regions of reticulocyte and B-lymphoblast ADA mRNAs from affected individuals were structurally indistinguishable from those of normal B lymphoblasts. Northern blot analysis performed under stringent hybridization and washing conditions confirmed a markedly increased amount of reticulocyte ADA mRNA in affected individuals as compared with controls. We conclude that the RBC-specific overexpression of ADA in this disorder occurs at the mRNA level.

MATERIALS AND METHODS

A 1.4-kb ADA cDNA sequence cloned into the HindIII site of pBR322 was provided by Dr Peter Daddona of Centocor, Malvern, PA. Dr Stuart Orkin of Children's Hospital in Boston provided oligonucleotide primers for ADA cDNA sequencing reactions as well as an Sp6 expression vector containing a 500-base pair (bp) ADA fragment spanning 347 bp of genomic sequence 5' to the cap site, exon 1, and 25 bp of exon 2. A 303 bp β-globin cDNA sequence cloned into pBluescript was provided by Dr Donald Rucknagel of the Comprehensive Sickle Cell Center, Cincinnati, OH. Vectors, enzymes, and primers for RNase mapping were obtained from Promega (Madison, WI). 32P-dCTP and 32P-GTP were purchased from Bethesda Research Laboratories, MD. Reverse transcriptase was obtained from Molecular Genetic Resources (Tampa, FL). Other enzymes and reagents used in cDNA cloning were purchased from New England Biolabs (Beverly, MA) and Pharmacia (Piscataway, NJ). Sequencing reagents were obtained from Amersham (Arlington Heights, IL). All other reagents were purchased from Sigma Chemical (St Louis).

Methods. Cell preparation and RNA extraction have been previously described. A cDNA library was constructed by the method of Okayama and Berg as modified by Gubler and Hoffman. Polyadenylated [poly (A)] proband reticulocyte RNA was selected from the Division of Hematology-Oncology, Department of Internal Medicine, University of Michigan Medical Center, and the Howard Hughes Medical Institute, Ann Arbor; and the Division of Hematology, Department of Medicine, Medical College of Albany, NY.

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by oligo (dT) chromatography. First-strand synthesis was carried out by AMV reverse transcriptase after denaturing with 10 mmol/L methyl mercury hydroxide and priming with oligo (dT)12-18. cDNA synthesis was performed using DNA polymerase I, Escherichia coli DNA ligase, and RNase H. Double-stranded DNA was blunt-ended by T4 DNA polymerase and ligated to synthetic phosphorylated EcoRI linkers. Free linkers were separated by EcoRI digestion and Sepharose CL-4B chromatography. The cDNA sequences were ligated into phosphorylated Agt1 DNA arms, packaged, and plated on E. coli host strain Y1088. The unamplified library was screened with a nick-translated 32P-labeled ADA cDNA probe. Seven clones were identified, plaque-purified, and subcloned into M13mpl8 phage. The 5'- and 3'-untranslated regions of all seven clones were sequenced from one strand by M13 universal primer. One clone was sequenced completely using synthetic oligonucleotide primers complementary to the anti-sense strand and spaced at 150 to 250 base intervals. Sequencing reactions were performed by the method of Sanger et al. RNase maps. RNase mapping was performed according to methods described by Melton et al. Schematics of vectors constructed for these studies are shown in Fig 1. A 441-bp XhoI-NcoI ADA genomic fragment containing the promoter, enhancer, and 94 bp of the first exon was spliced with a 59-bp NcoI-BamHI fragment of ADA cDNA containing the remaining 34 bp of exon 1 and 25 bp of exon 2. The resultant 500-bp fragment was subcloned into the BamHI and SalI sites of the Sp65 vector. The 324-bp 3' PstI-HindIII fragment of ADA cDNA was subcloned into a pGEM-3-blue vector. The β-globin vector was constructed by subcloning a 303-bp NcoI-EcoRI cDNA fragment into pGEM-3-blue. Each vector was linearized and transcribed in vitro from the Sp6 or T7 promoter to generate high-specific-activity, single-stranded, anti-sense RNA probes. Probes were hybridized overnight with 25 μg total cellular RNA at 60°C in buffer containing 80% formamide, 400 mmol/L NaCl, 40 mmol/L piperazine-N,N'-bis(Z-ethanesulfonic acid (PIPES) (pH 6.7), and 1 mmol/L EDTA. Hybrids were digested at 30°C in buffer containing 10 mmol/L Tris (pH 7.5), 5 mmol/L EDTA, 300 mmol/L NaCl, 40 μg/ml RNase A, and 2 μg/ml RNase T1. Digestion products were extracted with phenol:chloroform, precipitated with ethanol, washed with 70% ethanol, pelleted, dissolved in loading buffer, and run in 8 mol/L urea, 6% polyacrylamide gels. Gels were dried and autoradiographed with intensifying screens overnight at -70°C.

Northern blots. Northern blot analysis was performed according to the method of Maniatis et al. Ten micrograms of total cellular RNA from each sample was run in duplicate in 1% formaldehyde denaturing gels. DNA marker lanes were removed.
and stained with ethidium bromide. The gels were rinsed in 20× SSC, transferred overnight to nitrocellulose, and baked under vacuum for two hours. A lane containing MOLT-4 T-lymphoblast RNA was stained with methylene blue to determine the location of ribosomal subunit RNA. Blots were prehybridized in buffer containing 5% dextran, 5× SSPE, 5× Denhardt’s solution, 0.1% SDS, 150 μg/mL denatured salmon sperm DNA, and 35% (blot A) or 50% (blot B) formamide. Hybridization was performed overnight at 42°C in 15 mL prehybridization buffer with the addition of 1 × 10⁶ cpm/mL nick-translated 32P-labeled 1.4-kb ADA cDNA. Blots were washed with two changes each in 2× SSC-0.5% sodium dodecyl sulfate (SDS) for 10 minutes at room temperature, 2× SSC-0.5% SDS for 30 minutes at 55°C (blot A) or 68°C (blot B), and 0.1x SSC for 30 minutes at 55°C (blot A) or 68°C (blot B). Blots were autoradiographed overnight at ~70°C with intensifying screens.

RESULTS

cDNA cloning and sequencing. cDNA cloning and sequencing were performed to determine whether proband reticulocyte ADA mRNA had any structural abnormalities. The unamplified λ gt11 library contained 5 × 10⁶ recombinants, of which 1 × 10⁴ were screened. Seven clones were isolated and studied. Clone 3 extended from bp 14 through a 16-residue poly (A) tail. It was completely sequenced and matched exactly the exon sequences of the ADA gene as published by Wiginton et al. The 5’ termini of the remaining six clones were all within 2 to 21 bp of the transcription initiation site. Five clones had poly (A) tails ranging from 8 to 60 residues; the 3’ terminus of the remaining clone was at bp 1116. The 5’- and 3’-untranslated regions of all six clones were sequenced and were identical to clone 3. Portions of the coding regions of these additional six clones were also sequenced. Clone 2 contains the sixth intron; otherwise, no differences were found among these six clones, clone 3, and the referenced ADA sequence.

RNase protection experiments. RNase protection experiments were performed to determine whether alterations in the transcription initiation site or structural abnormalities might exist in the 5’ noncoding regions not covered by sequence data. The results are shown in Fig 2. The experiments shown in Fig 2A were performed with the 5’ ADA genomic probe described in the legend to Fig 1. On hybridization to normal ADA mRNA, the predicted RNase digestion product is a 153-bp protected fragment extending from the transcription initiation site through the 5’-untranslated region and translation initiation site and terminating within the second exon. A major band of the predicted size is present in RNA from MOLT-4 T lymphoblasts (lane 1), GM558 B lymphoblasts (lane 2), B lymphoblasts derived from the proband and an affected sister (lanes 3 and 4), and reticulocytes from the proband and sister (lanes 5 and 6). Several faint bands of higher molecular weights (mol wts) are also evident in lanes 1 through 6 and probably represent minor alternative transcription initiation sites, as has been noted by other researchers (reference 16 and R. Hirschhorn and S. Orkin, personal communication, March 1988). In contrast, no bands were detectable in RNA from normal reticulocytes (lanes 7 through 11).

RNase maps were repeated with a 379-nucleotide RNA probe generated from the 3’ coding and noncoding regions of ADA cDNA (Fig 1). Results are shown in Fig 2B. A band corresponding to the predicted 324-bp protected fragment was present in RNA from all lymphoblast cell lines and in reticulocyte RNA from affected individuals; again, no corresponding band was detected in normal reticulocyte RNA. Faint higher mol-wt bands in reticulocyte RNA samples reflected undigested probe. RNase mapping of MOLT-4 and reticulocyte RNA was repeated with a β-globin probe (Fig 1). Results (Fig 2C) demonstrated no hybridization to MOLT-4 T-lymphoblast RNA (lane 1) but did show the predicted 303-bp bands of similar intensity in RNA from all affected and normal reticulocytes (lanes 5 through 12). This study documents the presence of equivalent amounts of undegraded RNA in these experiments. These data also provide evidence that (a) there are no major differences in length or base composition of the 5’- and 3’-noncoding regions of ADA mRNA in B lymphoblasts and reticulocytes.
from affected patients as compared with those of normal B lymphoblasts, (b) the amount of ADA mRNA in affected reticulocytes is equal to or greater than the amount in B lymphoblasts from both patients and the normal control, and (c) the amount of ADA mRNA in affected reticulocytes is markedly increased over the amount in normal reticulocytes.

Northern blot analysis. Because the results of these RNase mapping experiments differ from those of previous Northern blot analyses,7 Northern blots were repeated under a variety of experimental conditions. Two Northern blots representing the least stringent and most stringent hybridization and washing conditions are shown in Fig 3. Blot A was hybridized at 42°C in buffer containing 35% formamide and washed at 55°C. All samples contained a faint band at ~4.4-kb and a more intense band at 2.0 kb, which correspond to the 28S and 18S ribosomal bands on a methylene-blue-stained control. An additional band was present at 1.5 kb in all lymphoblast RNA samples (lanes 1 through 4) and in reticulocyte RNA from the proband and the affected sister (lanes 5 and 6). This band did not appear in control reticulocyte RNA (lanes 7 through 9).

Blot B was hybridized in buffer containing 50% formamide and washed at 68°C. A single band appeared at 1.5 kb in all lymphoblast RNA samples (lanes 1 through 4) and in proband and sibling reticulocyte RNA (lanes 5 and 6). No band was evident in reticulocyte RNA controls (lanes 7 through 9). Hybridization to 28S and 18S ribosomal RNA was no longer apparent. Stripping and reprobing of both blots with β-globin cDNA demonstrated equal amounts of nondegraded reticulocyte RNA in lanes 5 through 9 (data not shown). These data confirm the quantitative difference in ADA mRNA in affected and control reticulocytes as shown in RNase protection experiments.

DISCUSSION

We previously showed that elevated erythrocyte ADA activity in this autosomal dominant hemolytic anemia is associated with a corresponding increase in immunoreactive ADA protein.7 Those studies supported the hypothesis set forth by earlier investigators that erythrocytes contained increased amounts of normal ADA protein.4 Cloning and sequencing of proband reticulocyte ADA cDNAs as well as RNase mapping experiments provide strong evidence that the defect is associated with increased amounts of structurally normal ADA mRNA in erythroid precursors. Because our initial investigations suggested that the defect occurred at the level of ADA mRNA translation, we isolated ADA cDNA clones from proband reticulocytes to examine potential regulatory sequences in the 5’- and 3’-noncoding regions. None of the seven clones contained any mutation in these regions, nor were alterations in length or base composition detected by RNase protection experiments. Clone 2 does contain a structural abnormality in the coding region; the 1.1-kb sixth intron has not been excised and appears in the mature mRNA. This region is not covered by the RNA probes used in protection experiments and is therefore not reflected in RNase maps. Failure to detect a 2.6-kb band on Northern blot analysis indicates that this mRNA is not a major component of ADA mRNA. Similar splicing intermediates in ADA cDNAs have been identified by other investigators.17

RNase maps also demonstrate quantitative differences in ADA mRNA in reticulocytes from affected individuals and controls that were not apparent in earlier Northern blot analyses but were clearly apparent on repeat analyses with more stringent hybridization and washing conditions. Binding of ADA cDNA probes to 28S ribosomal RNA on Northern blots has been noted by other investigators and attributed to hybridization to ADA mRNA precursors.18 Hybridization of the ADA cDNA probe to the 18S ribosomal RNA has not been previously reported. Areas of sequence similarity exist between ADA cDNA and 18S ribosomal RNA, including a stretch of identity of 30 of 39 residues between bases 534 and 572 of ADA cDNA.19 This homology, in conjunction with the vast molar excess of the ribosomal RNA sequences, may result in easily detectable and specific cross-hybridization.
Both Northern blot analysis and RNase mapping experiments demonstrate a markedly increased amount of ADA mRNA in reticulocytes from affected individuals. Because no bands are detected in up to 25 μg RNA from controls, the precise magnitude of this increase cannot be ascertained. RNase protection assays, however, detect an ADA band in as little as 250 ng proband reticulocyte RNA, indicating that the increase in ADA mRNA is at least 100-fold (data not shown).

The increase in reticulocyte ADA mRNA appears to be due to increased transcription of the ADA gene in nucleated erythroid precursors, loss of normal downregulation of ADA transcription during erythroid differentiation and/or maturation, or cell-specific alterations in posttranscriptional RNA processing. If the defect occurs at the transcriptional level, it may involve either a cis-altering regulatory mechanisms have been described.20-24 Posttranscriptional mechanisms such as RBC-specific changes in ADA mRNA transport and/or stability would be difficult to demonstrate in view of the technical problems of obtaining and culturing sufficient numbers of purified marrow erythroid precursors from affected individuals for study.

The role of ADA in erythropoiesis has not been studied. In investigating a Japanese kindred with a hemolytic anemia similar to that described here, Miwa et al showed that ADA activity and immunoreactive protein in cultured erythroblasts are approximately two times that which occur in mature RBCs.25 This study suggests that a marked decrease in ADA protein levels does not occur during normal erythroid development. However, the finding of low but significant elevations of ADA catalytic activity in Diamond-Blackfan syndrome supports the concept that expression of the ADA gene may be perturbed with aberrant maturation of the erythroid lineage.26

ADA activity in hematopoietic cells varies widely from <1 nmol/min/mg in mature RBCs to >1,000 nmol/min/mg in immature T lymphoblasts. These studies, in conjunction with our previous work,2 provide evidence that the amount of ADA-specific mRNA in B lymphoblasts and reticulocytes correlates with ADA activity and ADA immunoreactive protein. Because all hematopoietic cells are derived from a common progenitor, there appear to be specific control mechanisms for ADA expression as cells differentiate and mature. Further studies to define the mutation in ADA regulatory sequences or the altered erythrocyte gene product which mediates the erythrocyte ADA overproduction in this disorder should increase our understanding of the mechanisms controlling tissue-specific gene expression.

REFERENCES

3. Valentine WN, Paglia DE, Tartaglia AP, Gilsanz F: Hereditary hemolytic anemia with increased red cell adenosine deaminase (45- to 70-fold) and decreased adenosine triphosphate. Science 195:783, 1977
20. Lichtsteiner S, Wuarin J, Schibler U: The interplay of DNA-


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