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Correct Splicing Despite Mutation of the Invariant First Nucleotide of a 5′ Splice Site: A Possible Basis for Disparate Clinical Phenotypes in Siblings with Adenosine Deaminase Deficiency

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Summary

Adenosine deaminase (ADA) deficiency usually causes severe combined immune deficiency in infancy. Milder phenotypes, with delayed or late onset and gradual decline in immune function, also occur and are associated with less severely impaired deoxyadenosine (dAdo) catabolism. We have characterized the mutations responsible for ADA deficiency in siblings with striking disparity in clinical phenotype. Erythrocyte dAdo nucleotide pool size, which reflects total residual ADA activity, was lower in the older, more mildly affected sib (RG) than in her younger, more severely affected sister (EG). Cultured T cells, fibroblasts, and B lymphoblasts of RG had detectable residual ADA activity, while cells of EG did not. ADA mRNA was undetectable by northern analysis in these cells of both patients. Both sibs were found to be compound heterozygotes for the following novel splicing defects: (1) a $G^{+1} \rightarrow A$ substitution at the 5′ splice site of IVS 2 and (2) a complex 17-bp rearrangement of the 3′ splice site of IVS 8, which inserted a run of seven purines into the polypyrimidine tract and altered the reading frame of exon 9. PCR-amplified ADA cDNA clones with premature translation stop codons arising from aberrant pre-mRNA splicing were identified, which were consistent with these mutations. However, some cDNA clones from T cells of both patients and from fibroblasts and Epstein-Barr virus (EBV)–transformed B cells of RG, were normally spliced at both the exon 2/3 and exon 8/9 junctions. A normal coding sequence was documented for clones from both sibs. The normal cDNA clones did not appear to arise from either contamination or PCR artifact, and mosaicism seems unlikely to have been involved. These findings suggest (1) that a low level of normal pre-mRNA splicing may occur despite mutation of the invariant first nucleotide of the 5′ splice donor sequence and (2) that differences in efficiency of such splicing may account for the difference in residual ADA activity, immune dysfunction, and clinical severity in these siblings.

Introduction

Inherited adenosine deaminase (ADA) deficiency causes selective, but variable, immune deficiency (Giblett et al. 1972; Hirschhorn 1990; Hershfield and Mitchell, in press). Typical patients have severe combined immunodeficiency disease (SCID), with profound lymphopenia, absence of T- and B-cell function, failure to thrive, and life-threatening opportunistic infections from infancy. Ten to fifteen percent have a delayed clinical onset by age 6–24 mo and are usually diagnosed with SCID by age 2 years. Several late-onset patients, with less-severe childhood infections and gradual clinical and immunologic deterioration, have been diagnosed at age 4–35 years (Geffner et al. 1986; Levy et al. 1988; Santisteban et al. 1993; Shovlin et al. 1993). Finally, several immunocompetent individuals with sub-

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stantial ADA activity in nucleated cells have been identified in populations screened for erythrocyte ADA deficiency (partial ADA deficiency). Clinical phenotype correlates with overall impairment of deoxyadenosine (dAdo) catabolism, as reflected in dAdo nucleotide pool expansion and dAdo-mediated inactivation of S-adenosylhomocysteine hydrolase (SAHase) in erythrocytes (Morgan et al. 1987; Hirschhorn 1993; Santisteban et al. 1993; Hershfield and Mitchell, in press).

ADA deficiency is also marked by genetic heterogeneity: over 25 different ADA gene mutations have been identified (Hirschhorn 1993; Hershfield and Mitchell, in press; authors' unpublished observations). Sorting out the relationship between genotype and phenotype is complicated, because the disorder is rare, and although several mutations are recurrent, most patients are compound heterozygotes. In addition, environmental factors, including the nature of medical care, can play a large role in determining severity and age at diagnosis. The genetic background of patients may also influence phenotype by affecting resistance to some organisms, by predisposing to autoimmunity, or conceivably by affecting the residual level of ADA activity or alternative pathways of purine nucleoside metabolism. As with many genetic disorders, families with multiple affected individuals offer insight into the contribution of genotype to phenotype. However, with ADA deficiency, first-affected children are usually very ill by the time they are diagnosed, while affected sibs born afterwards are diagnosed and treated before the disease process has fully developed. Nevertheless, in several reported cases and in unpublished cases with which we are familiar, impairment of lymphocyte function has been similar in sibs (Giblett et al. 1972; Hirschhorn 1979; Rubinstein et al. 1979; Markert et al. 1987*a*; Shovlin et al. 1993).

In light of this background, it was of interest to find sisters with strikingly dissimilar clinical presentations, in whom the usual pattern of severity was reversed (Umetsu et al. 1994). The second-born child, EG, presented first with serious infections and failure to thrive at age 4 mo; she was diagnosed with SCID and ADA deficiency at age 9 mo, during hospitalization for *Pseudomonas* sepsis and *Pneumocystis* pneumonia. At diagnosis, T lymphocytes were profoundly decreased, lymphocyte responses to mitogens were <10% of normal, and, despite immunization, specific antibody to tetanus and diphtheria was undetectable. Her healthy 39-mo-old sister, RG, was then tested and found to be ADA deficient. She had an unremarkable history, including normal development (weight in 97th percentile) and uncomplicated varicella zoster at age 6 mo. Although she was lymphopenic, antibody production, delayed hypersensitivity, and in vitro T-cell function were intact. She became more lymphopenic over a period of 6–7 mo and developed persistent upper-respiratory infections. Along with her sister, she was then treated by enzyme replacement with polyethylene glycol (PEG)-ADA (Umetsu et al. 1994).

The disparate clinical phenotypes in these sibs raised the possibility of an unusual genetic basis for their disorder. Here we report that the children are compound heterozygotes for two splicing mutations—one is a rearrangement that disrupts the polypyrimidine tract of a 3′ splice site, and the other is a point mutation of the invariant G^{+1} nucleotide of a 5′ splice donor sequence. Our findings indicate that some normal pre-mRNA splicing may occur despite this latter mutation and suggest that variation in splicing efficiency of this mutant allele may account for phenotypic variability in this family.

Material and Methods

Cell Culture

T cells were cultured from peripheral blood mononuclear cells as described by Arredondo-Vega et al. (1990), except that culture medium contained 15% heat-inactivated (30 min, 56°C) fetal bovine serum (GIBCO) and 50 U/ml IL-2 (Boehringer Mannheim), instead of T-cell–conditioned medium. ADA activity in extracts of these cells, fibroblasts, and EBV-transformed B-cell lines were determined as described elsewhere (Arredondo-Vega et al. 1990).

Analysis of ADA Gene Mutations

Standard procedures were used for subcloning and restriction-enzyme analysis (Sambrook et al. 1989) and for PCR (Erlich 1989; Innis et al. 1990); and recommendations of the suppliers of reagents used in these procedures were followed. Wild-type ADA cDNA and genomic sequences are as reported elsewhere (Wiginton et al. 1984, 1986). ADA cDNA sequences are numbered relative to the start of translation, and genomic DNA is numbered according to Wiginton et al. (1986). Specific primers for PCR amplification of full-length ADA cDNA and segments of the cDNA and of ADA genomic DNA have been described elsewhere (Santisteban et al. 1993). In addition, primers for amplifying a 638-bp genomic DNA segment spanning the IVS 2/exon 3 junction were 5′CACTCACCAGCTGCGATTAA (sense; bp 14948-14967) and 5′AGGAGGACAAGACTCA-

Table 1

ADA Activity in Cultured Cells

	ADA Activity (nmol/h/mg protein)		
	EG	RG	Normal
Cultured T cells	7.3, 10	48, 168.6	2,047 ± 1,360[a]
EBV B-cell line	<10	16.9, 40.4	7,435 ± 3,285[b]
Fibroblasts	19.2	120.2, 103.8	758

NOTE.—Where two values are shown, they represent independent determinations.

[a] Arredondo-Vega et al. 1990.

[b] Markert et al. 1987*b*.

GAGGC (antisense; bp 15585-15565). Primers for amplifying a 184-bp genomic segment spanning the IVS 8/exon 9 junction for heteroduplex analysis (see Results) were 5'TATAGAGAGGCAGAAAGCAGGG (sense; bp 28793-28814) and 5'TGACTGCATGCTCCGTGT (antisense; bp 28977-28958). PCR products were subcloned into pUC18 or into the TA cloning vector (Invitrogen). Double-stranded DNA was sequenced with ^{35}S-dATP (Amersham) using Sequenase (USB, Cleveland). After gel purification, uncloned genomic DNA PCR products were sequenced using the Cycle Sequencing kit (BRL), with ^{32}P-end-labeled sequencing primers. Northern analysis of ADA and SAHase mRNA was performed as described elsewhere (Arredondo-Vega et al. 1990) by using probes prepared from pADA211 (Wiginton et al. 1983) and pDEC16-1 (Coulter-Karis and Hershfield 1989).

Allele-specific oligonucleotide (ASO) hybridization analysis of PCR-amplified cDNA clones was performed essentially as described elsewhere (Arredondo-Vega et al. 1990), with the following ^{32}P-end-labeled probes: (*a*) normal exon 2/3 junction, 5'CTATGGCAG|GAGGAGAGG; (*b*) 4-bp insertion at the exon 2/3 junction, CTATGGCAGataa|GAGGAG; (*c*) normal exon 8/9 junction, CACTTCGAG|ATCTGCCCC; and (*d*) deletion of exon 9 (exon 8/10 junction), CACTTCGAG|GCTCAAAAA (| indicates exon junction, and lowercase letters indicate inserted nucleotides).

Results

Metabolic Studies

EG and RG each had ~0.5%–1% of normal erythrocyte ADA activity, and each parent had approximately one-half of normal erythrocyte ADA activity (Umetsu et al. 1994). ADA activity was essentially undetectable in cultured T cells, B lymphoblasts, and fibroblasts from EG, the more severely affected child, but was present at a low level in cells from her older sister, RG (2%–8% in T cells) (table 1). The residual ADA activity in RG's cells was completely inhibited by 2 μM deoxycoformycin (data not shown). The level of total dAdo nucleotides (dAXP) in pretreatment red cells was correspondingly lower in RG (175 nmol/ml; range 123–202) than in EG (269 nmol/ml; normal <2 nmol/ml) (Umetsu et al. 1994). By comparison, erythrocyte dAXP ranged from 350 to >1,800 nmol/ml in 12 patients with SCID diagnosed at <1 year of age, compared with 60 to ~300 nmol/ml in 7 patients with late/delayed-onset SCID, who were 1.5–15 years old at diagnosis (Santisteban et al. 1993; Hershfield and Mitchell, in press). Red-cell SAHase activity was decreased to 0.37 nmol/h/mg for EG and 0.43 nmol/h/mg for RG (normal 4.2 ± 1.9 nmol/h/mg).

Analysis of ADA Mutations

ADA mRNA was undetectable by northern analysis in cultured T cells of both children, while SAHase mRNA was similar to that of the control (fig. 1). Findings were the same with fibroblasts and EBV-transformed B cells of both patients (data not shown). However, it was possible to amplify ADA cDNA from

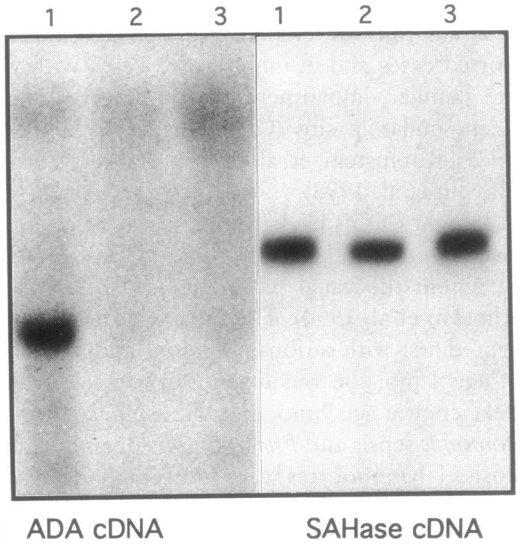

Figure 1 Northern blot analysis of total cellular RNA from cultured T cells. The blot was probed with ADA cDNA (*left*), then washed and reprobed with SAHase cDNA (*right*). Lane 1, Control. Lane 2, EG. Lane 3, RG.

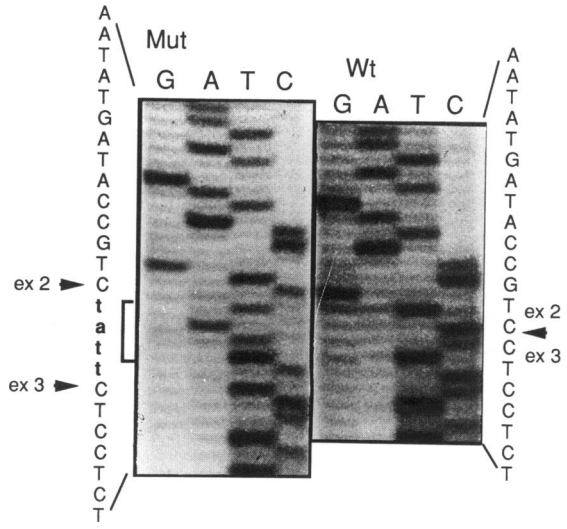

Figure 2 Sequence (antisense) of PCR-amplified cDNA subclones derived from T cells of patient EG. *Left,* (Mut) clone, which contains a 4-bp insertion at the exon 2/3 junction. *Right,* (Wt) clone with normal sequence.

reverse-transcribed RNA prepared from all three cell types from RG; in the case of EG we were successful only with T cells. Sequencing of several subcloned PCR products revealed two mutant cDNAs. The first had a 4-bp insertion, ATAA, at the exon 2/3 junction (fig. 2). The second lacked exon 9, with precise joining of exons 8 and 10 (see below). On the basis of these results, we amplified and analyzed segments spanning these regions from genomic DNA of the patients and their parents.

Direct sequencing of the amplified exon 2/IVS 2 junction showed the children to be heterozygous for a G→A transition at bp 15357 (numbered as in Wiginton et al. 1986), the first position of IVS 2 (G^{+1}). This change eliminates a *Bsp*MI restriction site. On *Bsp*MI digestion of an amplified genomic segment spanning the exon 2/IVS 2 junction (which normally contains two *Bsp*MI sites), both patients and their mother were found to be heterozygous, while their father gave only the normal pattern (fig. 3). As discussed further below, G^{+1}→A intronic mutations disrupt normal splicing, causing exon skipping or activation of cryptic splice donor sites (Treisman et al. 1983*b;* Wieringa et al. 1983; Kuivaniemi et al. 1990). In the present case, use of a nonconsensus splice site at bp +5 of IVS 2 accounts for inclusion of the first four intronic bp in mRNA (CAG/gtaagt→CAGataa/gtccat). As a result, amino acids 32–36 are predicted to change from RRRGI to RX, where X is a new TAA stop signal, which should eliminate 330 C-terminal residues and result in a nonfunctional protein. Nonsense codons also often result in marked destabilization of the corresponding mRNA (Losson and Lacroute 1979; Baumann et al. 1985; Urlaub et al. 1989). We have reported another ADA-deficient patient in whom a G^{+1}→A mutation in IVS 10 caused a similar 4-bp insertion and a markedly reduced level of message for that allele (Santisteban et al. 1993).

Sequencing of a subcloned genomic PCR fragment spanning exon 9 and its flanking introns revealed both patients to be heterozygous for a complex rearrangement in which the last 3 bp of IVS 8 and first bp of exon 9 (cagA) are replaced by a 17-bp insertion (net change, +13 bp) (fig. 4, *top*). A 14-bp inner segment of the insertion, bounded 5′ by TG and 3′ by G nucleotides, consists of two elements closely related to sequences in the normal splice region (indicated by arrows in fig. 4, *bottom*). The first (5′) 9-bp element repeats a segment of the polypyrimidine tract but with strand inversion and reversal of 5′-to-3′ orientation. The second element, which overlaps the 3′ end of the first, is an 8-bp palindrome found at the 3′ end of the normal splice region,

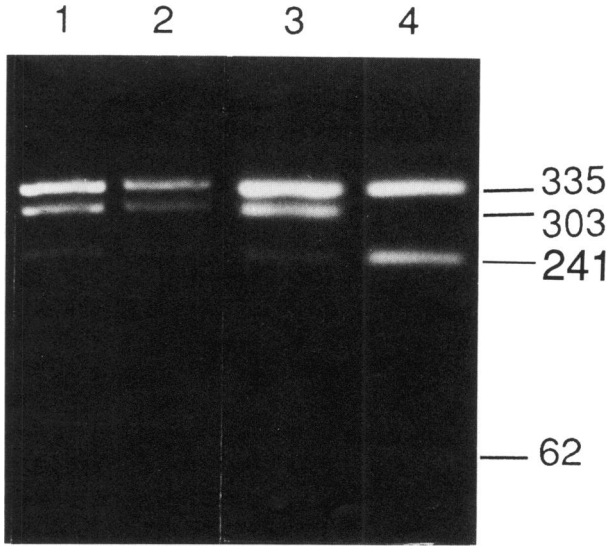

Figure 3 Inheritance of the IVS 2 G^{+1}→A mutation. A 638-bp PCR fragment spanning the IVS 2/exon 3 junction was generated from genomic DNA and digested with *Bsp*MI restriction endonuclease (New England BioLabs). Digestion products were analyzed by electrophoresis on 3% agarose gels and stained with ethidium bromide. Products of 335, 241, and 62 bp are expected for the wild-type allele, and products of 335 and 303 bp are expected for the IVS 2G^{+1}→A mutant allele. Lane 1, RG. Lane 2, EG. Lane 3, Mother. Lane 4, Father.

which includes the deleted cagA. These features suggest an error during replication, which may have been initiated by template-strand switching or "loopback" copying of the primer strand, introducing the 4-bp deletion and the inverted first element. Return to the original template strand would produce the second element. The TG and G nucleotides bounding the insertion may have been introduced during realignments of the growing point. Regardless of its origin, by disrupting the polypyrimidine tract with a string of 7 purine nt, the IVS 8 rearrangement can account for cDNA clones missing exon 9 (see Discussion). Loss of exon 9 changes the reading frame after codon 260, generating a TGA

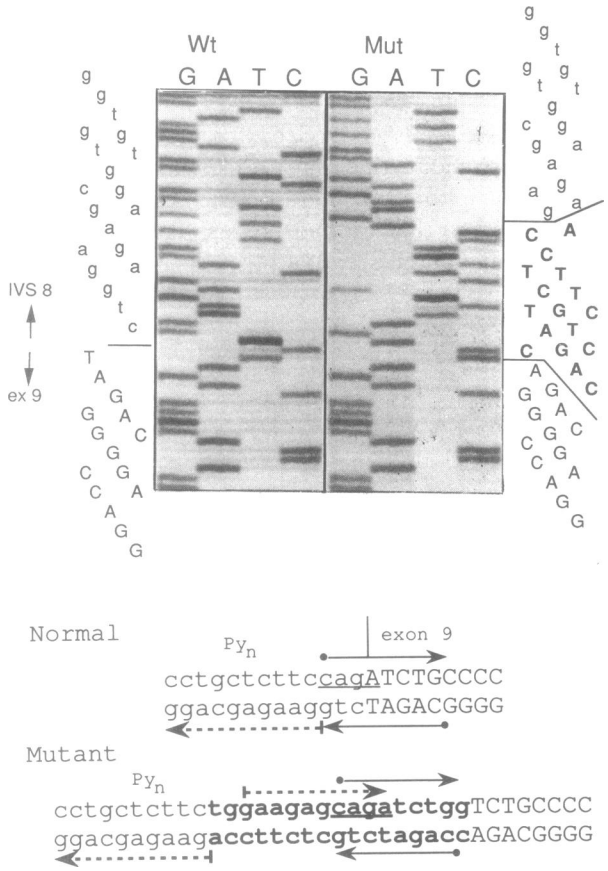

Figure 4 *Top*, Sequence (antisense) of the IVS 8/exon 9 junction in PCR-amplified genomic DNA subclones derived from cultured T cells of patient RG. *Left* (Wt), clone with normal sequence; *Right* (Mut) clone with insertion/rearrangement. Nucleotides found in the normal exon are upper case; normal intron, lower case; nucleotides in the insertion are bold. *Bottom*, Sequence elements of normal and rearranged IVS 8/exon 9 junctions. Py_n = polypyrimidine tract. Arrows indicate 5′→3′ orientation of elements present at the normal junction and in the insertion (for details, see Results). Inserted nucleotides are boldface.

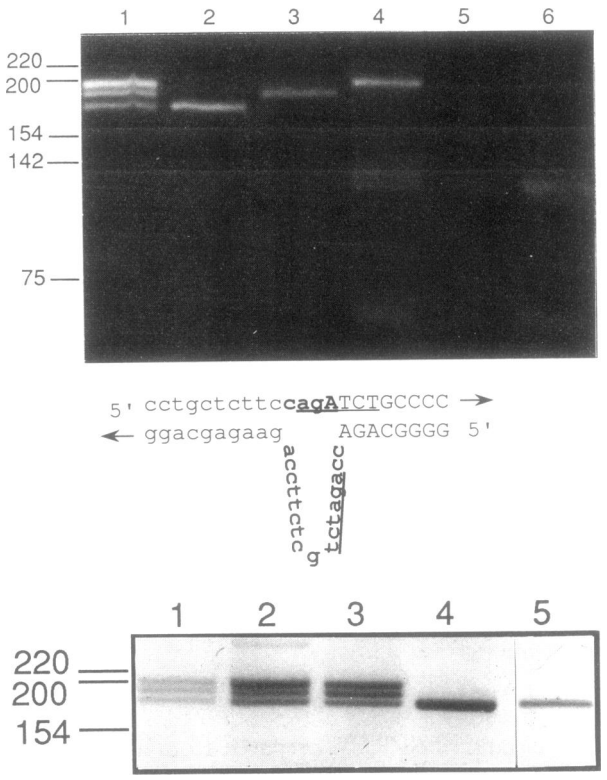

Figure 5 *Top*, Ethidium bromide–stained agarose gel analysis of PCR-amplified genomic DNA fragments spanning the IVS 8/exon 9 junction. Lanes 1 and 4, Patient EG (uncloned, amplified from blood DNA). Lanes 2 and 5, Genomic subclone bearing the normal junction. Lanes 3 and 6, Genomic subclone bearing the rearranged junction. Lanes 1–3, undigested PCR products. Lanes 4–6, Digestion with *Bgl*II restriction endonuclease (BRL). *Middle*, Alignment of base-paired and unpaired (boldface) segments in heteroduplex formed between PCR products of the homologous IVS 8/exon 9 junctions derived from normal and rearranged ADA alleles. *Bgl*II restriction endonuclease recognition sequences are underlined. Nucleotides in normal exon are upper case, and nucleotides in intron are lower case. *Bottom*, Inheritance of IVS 8/exon 9 genomic rearrangement. Genomic DNA samples were amplified and analyzed directly by agarose gel electrophoresis as in top panel. Lane 1, RG. Lane 2, EG. Lane 3, Father. Lane 4, Mother. Lane 5, Normal control.

stop signal four triplets downstream. The predicted consequences are loss of 100 C-terminal amino acids and reduced mRNA stability.

Heteroduplexes formed from products of homologous loci during PCR reactions can be used to identify heterozygotes carrying deletion/insertion mutations (Nagamine et al. 1989). We used this phenomenon to define the inheritance of the IVS 8 rearrangement. Amplification of the involved region from genomic DNA of the children and their father gave three PCR prod-

ucts, while that of their mother and a control gave one (fig. 5, *top* and *bottom*). The lowest and middle bands correspond to the PCR products obtained using as templates cloned genomic DNA segments bearing the normal and IVS 8 insertion sequences, respectively (fig. 5, *top,* lanes 1–3). Each of these lower bands was sensitive to digestion with *Bgl*II (fig. 5, *top,* lanes 5 and 6), as expected, since this restriction site (AGATCT) is present at the normal IVS 8/exon 9 junction and within the insertion (fig. 4). The top band (the putative heteroduplex) was resistant (fig. 5, *top,* lane 4). The retarded migration and resistance to digestion are presumably due to unpaired regions on both strands of the heteroduplex, which include part or all of the *Bgl*II sites (fig. 5, *middle*). To confirm that the top band is a heteroduplex, the upper band remaining after *Bgl*II digestion (fig. 5, *top,* lane 4) was reamplified with the original PCR primers, regenerating all three bands (data not shown).

Other ADA cDNA Species, Including Wild Type

In our initial screening of ADA cDNA clones we found some that lacked mutations in exons 2, 3, and 9. We analyzed the junctions of exons 2/3 and 8/9 of additional PCR-generated cDNA subclones derived in several independent experiments from RG's fibroblasts, T cells, and B lymphoblasts and from EG's T cells (we were unsuccessful in amplifying ADA cDNA from EG's fibroblasts and B lymphoblasts). Of 56 clones analyzed by sequencing or ASO hybridization, one-third had changes either clearly or probably related to the genomic mutations: 11 (19.6%) lacked exon 9, and 5 (8.9%) had the 4-bp insert at the exon 2/3 junction. Single clones (1/56 or 1.8% each) lacked exon 2 entirely or had a deletion of the first 3 bp, GAG, of exon 3, which may have arisen from aberrant selection of the 3′ splice site in IVS 2 (see Discussion). Of most interest, 38/56 (66.7%) clones had no changes in exons 2, 3, or 9. The coding regions of several of these clones were sequenced. In some, isolated point mutations, presumably PCR artifacts, or aberrant splicing of exon 7, known to occur in normal individuals (Akeson et al. 1989), were found but were considered irrelevant. However, several clones had normal coding sequences, including six derived from RG's T cells, one from RG's fibroblasts, and two from EG's T cells.

PCR-mediated recombination (Meyerhans et al. 1990; Marton et al. 1991) is a potential artifact that might have given rise to normal cDNA. However, the majority of the clones analyzed from both patients showed normal splicing at both sites, while none were mutant at both sites. The absence of any reciprocal product makes recombination unlikely. In addition, we detected no normal recombinants in control experiments in which mixtures of cloned cDNAs bearing the exon 9 deletion or the 4-bp insert at the exon 2/3 junction were used to prime PCR reactions under conditions similar to those used for reverse transcription–PCR (data not shown). We were also concerned about possible contamination, either with normal cells during tissue culture or, despite precautions and controls, with normal ADA cDNA during PCR amplification. Therefore, we repeated our studies with a short-term culture of T cells established from a fresh blood sample from patient RG. Total RNA isolated from these cells was incubated in first-strand cDNA reactions with or without reverse transcriptase (*−RT* control). Aliquots of these incubations, or buffer (*−template* control), were used to prime PCR reactions for amplifying the ADA coding region. The products of these reactions were tested by slot-blot hybridization to ^{32}P-labeled ASO probes for the wild-type and mutant cDNA sequences at the exon 2/3 and exon 8/9 junctions (fig. 6*A*). The uncloned cDNA product derived from RG's T cells gave positive signals with all four ASO probes, while the *−RT* and *−template* controls showed no hybridization with any probe; positive controls (cloned normal and mutant ADA cDNAs) reacted only with the appropriate probes (data not shown). After subcloning, seven random colonies were analyzed by ASO hybridization: 5/7 wild type and one of each mutant type were found (fig. 6*B*). In two independent experiments with EG's T cells (derived from blood samples obtained on different occasions), we obtained similar results: in one experiment, two of five, and in the second, seven of eight, clones analyzed showed normal splicing at both sites.

Discussion

To summarize, both sisters are compound heterozygotes for the same two novel ADA mutations. One allele, inherited maternally, had a G→A transition at position +1 of IVS 2; the other, inherited from the father, had an unusal complex rearrangement of the IVS 8/exon 9 junction. The former mutation disrupts a 5′ splice donor site, causing either exon 2 skipping or a 4-bp insertion at the exon 2/3 junction because of use of a cryptic splice site; the latter disrupts a 3′ splice acceptor site, resulting in skipping of exon 9. By introducing nonsense codons in the aberrantly spliced mRNA species, these mutations account for the absence of detectable of ADA mRNA on northern analy-

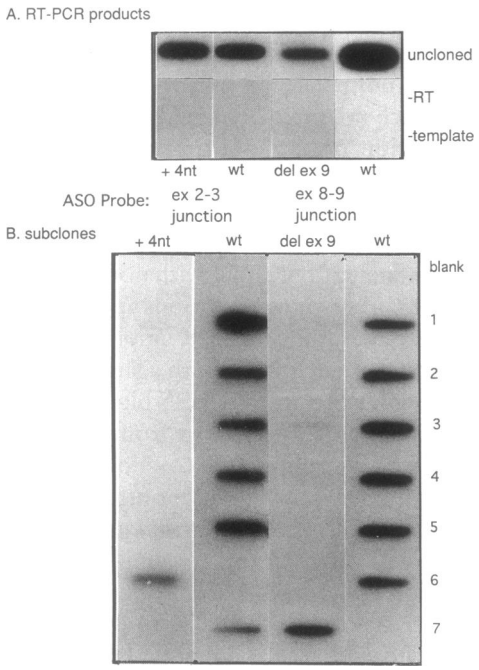

Figure 6 ASO analysis of the exon 2/3 and exon 8/9 junctions in ADA cDNA. *A*, First-strand cDNA was synthesized from total RNA prepared from RG's T cells (harvested on day 19 of culture); a control reaction contained this RNA but not reverse transcriptase (*−RT*). Aliquots of both reactions and a buffer blank (*−template*) were used to prime PCR reactions with full-length ADA cDNA primers (Santisteban et al. 1993). After 30 cycles, aliquots of these reactions were used to prime a second round of amplification, with nested primers for the ADA coding region (Santisteban et al. 1993). Aliquots of these last reactions were tested by slot blot hybridization to ^{32}P-labeled ASO probes for the normal or mutant exon junctions (see Material and Methods). *B*, ASO analysis of subcloned PCR products derived from RG's reaction described in panel *A*.

sis of T cells, fibroblasts, and B-cell lines of both patients. In combination with the predicted truncation of the encoded proteins by 330 and 100 amino acids, these mutations would be expected to eliminate ADA activity completely. However, cultured cells of the less severely affected patient, RG, had detectable ADA activity, and both patients had relatively modest erythrocyte metabolic abnormalities, suggesting a residual low level of ADA activity in some tissue(s) in vivo. Consistent with the latter findings but not easily reconciled with either genomic mutation is the reproducible ability to generate normally spliced ADA cDNA from T cells of both patients and from fibroblasts and B lymphoblasts of RG.

Before we discuss the possibility of correct splicing of pre-mRNA derived from one of the patients' mutant alleles, two other sources of the normal cDNA clones must be considered. First, cells expressing normal ADA mRNA might have arisen by engraftment of placentally transferred maternal lymphoid cells. Isolation of normal cDNA from fibroblasts, as well as from RG's lymphoid cells, makes this explanation unlikely. Second, mosaicism, established by analysis of EBV-transformed B-cell lines, has recently been identified in a late-onset ADA-deficient patient who recovered spontaneously (Hirschhorn et al. 1993). In that case parental inheritance was apparently not established (Bonthron et al. 1985; Hirschhorn et al. 1993). It was thus possible that a forward somatic cell mutation in the patient caused early expression of an ADA-deficient phenotype, which resolved with gradual selection for cells with one normal ADA allele. In the present sibs, both ADA mutations were parentally transmitted. Somatic cell reversion of one of the mutations (presumably the point mutation in IVS 2) might have resulted in mosaicism. However, normal cDNA was isolated from both patients. The probability that reversion, a very rare event, would occur independently in both sibs must be considered extremely low. In addition, we analyzed 24 EBV-transformed B-cell clones from the more mildly affected sib, RG, and found none that expressed a high level of ADA activity (data not presented).

Although there is considerable variation, the consensus mammalian 3′ splice region consists of a highly conserved C/TAG, preceded by any nucleotide and then a pyrimidine-rich tract (Py_n, where $n \geq 12$ bp); the branch point is usually located 18–40 bp upstream from the splice site (Mount 1982; Ohshima and Gotoh 1987; Shapiro and Senapathy 1987). These elements are present in the rearranged 3′ splice junction of IVS 8; however, a purine-rich tract is interposed between the CAG and Py_n, and the 5′ end of exon 9 is altered (fig. 4). Studies of model splicing substrates, including some with a purine-rich fragment placed between the CAG and Py_n, indicate an important role for Py_n in spliceosome assembly and selection of the branch point and 3′ splice site (Wieringa et al. 1984; Frendewey and Keller 1985; Ruskin and Green 1985; Reed 1989; Roscigno et al. 1993). Consistent with these results, splicing of mRNA derived from the mutated IVS 8 allele was probably abolished. Thus, we identified 12 cDNA clones lacking exon 9, but we found none that spliced after the CAG (apparently the only potential 3′ splice site in the immediate vicinity of the exon 9 junction). The reading frame of such an mRNA would be altered after codon 261, with a new stop signal at codon 311. Regardless of whether any undetected aberrant splicing of IVS 8 oc-

curs, it seems virtually impossible that a normal mRNA could be derived from this allele.

The consensus pre-mRNA 5′ splice sequence is CAG-guaagu, with the first two intron positions (underlined) being essentially invariant (Breathnach and Chambon 1981; Mount 1982; Padgett et al. 1986; Ohshima and Gotoh 1987; Shapiro and Senapathy 1987). Substitutions at G^{+1} allow cleavage at the mutant splice junction to occur, but with reduced efficiency, and the abnormal lariat-3′ exon structure formed is a "dead-end" intermediate. The second splicing reaction, cleavage at the 3′ splice site with exon joining, is blocked (Newman et al. 1985; Parker and Guthrie 1985; Reed and Maniatis 1985; Aebi et al. 1986, 1987; Lamond et al. 1987; Séraphin et al. 1988; Séraphin and Rosbash 1990), possibly because the spliceosome must recognize a normal branch-point structure (G [2′–5′] A) before splicing can proceed (Séraphin et al. 1988; Séraphin and Rosbash 1990; Green 1991). Mutations of G^{+1} of mammalian and yeast pre-mRNA introns have been shown to activate cryptic splice site(s) or result in skipping of the preceding exon, but normal splicing has not been observed (Newman et al. 1985; Parker and Guthrie 1985; Reed and Maniatis 1985; Aebi et al. 1986, 1987; Lamond et al. 1987; Séraphin et al. 1988; Séraphin and Rosbash 1990). Of 29 reports of G^{+1} mutations in patients with genetic disorders, splicing products were examined in 14; in none was normal splicing detected (Krawczak et al. 1992).

We observed both exon skipping and cryptic splice site activation due to the IVS 2 $G^{+1}\rightarrow$A mutation in our patients. However, we would like to consider the possibility that normal splicing of IVS 2 also occurred, perhaps because the block in the second step of splicing is leaky to a slight degree. This speculation is based partly on the finding of a few immunoglobulin introns that do not have a G in position +1 (Shapiro and Senapathy 1987) and, by analogy, on the recent finding that self-splicing group II introns bearing G^{+1} mutations undergo normal splicing, although with much reduced efficiency (Peebles et al. 1993). Group II introns possess a conserved 5′ splice sequence and a splicing pathway similar to pre-mRNA introns and are considered evolutionary precursors of pre-mRNA introns and spliceosome RNA components (Sharp 1985). We also found a single cDNA clone with a deletion of the first 3 bp of exon 3. This may have resulted from activation of a nonconsensus cryptic 3′ splice site; that is, instead of . . .cag/GAGGAGAGG, cleavage may have occurred after the second AG dinucleotide, . . .cagGAG/GAGAGG (underlined bp deleted from mRNA). If so, this indicates again that the block in the second splicing step caused by the G^{+1} mutation may not be absolute. It also suggests that G^{+1} mutations of the 5′ splice site may affect 3′ splice site selection, perhaps as a consequence of an interaction of the abnormal branch-point structure with the splicing machinery.

The majority of cDNA clones we analyzed had normal exon 2/3 and exon 8/9 junctions. This probably reflects the much greater stability of normal mRNA than of aberrantly spliced species possessing nonsense mutations. From northern analysis, the absolute level of normal mRNA must be very low. It was detected because no more-abundant ADA mRNA species were present in the cells of our patients to compete in reverse transcriptase and PCR reactions. Such competition (as in studies of four $G^{+1}\rightarrow$A COL3A1 gene mutations in heterozygotes with Ehlers-Danlos syndrome type IV [Cole et al. 1990; Kuivaniemi et al. 1990]) or use of standard cDNA cloning methods prior to availability of PCR may account for the failure to detect normally spliced products of disease-causing G^{+1} mutations. It is also possible that factors related specifically to the structure of ADA IVS 2, which consists of 7,052 bp, may account for some normal splicing in the present case. Selective metabolic pressure for expression of ADA activity in some cell lineages may also have played a role, favoring survival of cells that expressed some normally spliced ADA mRNA.

The finding of a low level of normal ADA mRNA expression can explain the residual ADA activity, relatively limited erythrocyte dAXP pool expansion, and mild clinical and immunological phenotype of patient RG. In light of the very low absolute levels of ADA mRNA, quantitating the difference in expression of normal ADA mRNA in cells of EG and RG would be technically very difficult, and results with cultured cells may not reflect tissue levels in vivo. However, we consistently amplified ADA cDNA from RG's T cells, fibroblasts, and B lymphoblasts—but only from EG's T cells —suggesting that less efficient expression of normal ADA mRNA might account for EG's more severe enzyme deficiency, immune dysfunction, and clinical phenotype (Umetsu et al. 1994). Tissue- and patient-specific variation in splicing efficiency were proposed to account for a remarkable difference in clinical severity among family members and different ethnic groups with an unusual exonic splicing mutation in the HexB gene (McInnes et al. 1992).

Other factors may have contributed to EG's disease severity. At age 30 mo, she was discovered to have an

abdominal mass and markedly elevated serum α-fetoprotein, a marker for hepatoblastoma. This diagnosis was established; in spite of chemotherapy and resection, the malignancy has since proved fatal. Tumor cells showed trisomy 20 and 2, a karyotype typical for hepatoblastoma (Fletcher et al. 1991; Sokup and Lampkin 1991); karyotype of blood lymphocytes was normal (C.-L. Hsieh and Mark J. Pettenati, unpublished information). Analysis of stored plasma samples from EG (obtained for monitoring plasma PEG-ADA activity) showed that α-fetoprotein had been elevated (3,000 ng/ml; normal <15 ng/ml) at the time EG was diagnosed with ADA deficiency (age 10 mo). Hepatoblastoma is a rare tumor that apparently arises in primitive embryonic hepatic cells that persist abnormally after birth. It is not associated with ADA deficiency or other primary immunodeficiencies, and there is no known relationship of therapy with PEG-ADA to any malignancy. It is possible that the process that led to the karyotypic changes and tumor development is related to the genesis of the unusual complex rearrangement of IVS 8, which seems to involve an interesting replication error (see Results). Occult tumor present during the first months of life may have increased the severity of immune deficiency or its consequences in EG. However, progression to massive enlargement following diagnosis did not interfere with response to PEG-ADA therapy. During the 20 mo prior to her presentation with tumor, EG resumed normal growth, became resistant to infection, and developed specific immune function (Umetsu et al. 1994).

Nearly all of the ADA mutations reported to date have been from patients with SCID or from healthy children with partial ADA deficiency. Of >25 different mutations identified in these groups, only 2 were splicing defects (Akeson et al. 1987; Kawamoto et al. 1993). In contrast, we have identified five novel splicing defects among eight families with delayed/late-onset ADA deficiency (Santisteban et al. 1993; and present report), and a sixth has recently been reported in another patient with mild disease (Hirschhorn et al. 1993). Our present findings, and those in other delayed/late-onset patients (Santisteban et al. 1993) suggest that, in ADA deficiency, as in other genetic disorders (Treisman et al. 1983*a*; Krainer et al. 1984; Kishimoto et al. 1989; McInnes et al. 1992), the efficiency of alternative splice site selection may be an important mechanism underlying mild or variable clinical phenotypes. Recent efforts that might result in therapies aimed at enhancing normal splicing are thus of considerable interest (Dominski and Kole 1993).

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