Spontaneous *in vivo* reversion to normal of an inherited mutation in a patient with adenosine deaminase deficiency

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Somatic mosaicism in genetic disease generally results from a *de novo* deleterious mutation during embryogenesis. We now describe a somatic mosaicism due to the unusual mechanism of *in vivo* reversion to normal of an inherited mutation. The propositus was an adenosine deaminase-deficient (*ADA*$^-$) child with progressive clinical improvement and unexpectedly mild biochemical and immunologic abnormalities. Mosaicism due to reversion was evidenced by absence of a maternally transmitted deleterious mutation in 13/15 authenticated B cell lines and in 17% of single alleles cloned from blood DNA, despite retention of a maternal 'private' *ADA* polymorphism linked to the mutation. Establishment of significant somatic mosaicism following reversion to normal could modify any disorder in which revertant cells have a selective advantage.

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Deficiency of the purine salvage enzyme adenosine deaminase (ADA) results in a rare, autosomal recessive immunodeficiency disorder of varying severity. In general, the degree of retention of residual ADA activity correlates inversely with both clinical severity and extent of accumulation of the toxic metabolites deoxyadenosine and deoxy ATP. Individuals with mutations at the *ADA* locus that result in complete absence of the enzyme exhibit neonatal onset of severe combined immunodeficiency (SCID), a disorder that, in the absence of therapy, is lethal prior to two years of age. Mutations resulting in retention of 1–5% of ADA activity are associated with progressive immunodeficiency including a delayed infantile onset immunodeficiency with retention of humoral (B cell) immunity, a later childhood onset immunodeficiency and an adult onset disorder that is clinically indistinguishable from the relatively frequent syndrome common variable immunodeficiency. All of these patients accumulate considerable amounts of deoxy ATP in erythrocytes and excrete markedly elevated amounts of deoxyadenosine. Individuals who totally lack ADA in erythrocytes, but retain residual ADA ranging from 10–80% of normal in non-erythroid cells and only minimally accumulate deoxyadenosine and deoxy ATP, have been ascertained by screening of healthy populations or by newborn screening. A number of such individuals, originally designated as 'partially' ADA deficient, have remained healthy into early adult life (refs 1–7; unpublished data).

It is well recognized that somatic mosaicism due to *de novo* mutations during embryogenesis is an additional basis for milder phenotype in many X-linked and autosomal dominant inherited disorders[8]. We have previously described somatic mosaicism as a modifier of phenotype in a young adult who presented at two years of age with complete ADA deficiency and immunodeficiency but who is currently alive at 20 years of age without therapy[9]. Parental DNA was unavailable, and we therefore could not determine whether or not somatic mosaicism had resulted from the usual mechanism of a *de novo* mutation on a normal allele during embryogenesis.

We have now studied another patient from an unrelated family who also had an unexpectedly mild phenotype. A prior sib had presented at 14 months with classic delayed onset SCID and died of the disorder before three years of age. By contrast, the sib studied here had a progressively milder course and is currently essentially normal without therapy at over 12 years of age (ref. 10 and case history). We now show that he is a somatic mosaic for a missense mutation (Arg156His), previously identified as deleterious in several unrelated *ADA*$^-$ immunodeficient patients[11]. The mutation was heteroallelic with a splice site mutation (IVS5 +1 GT to AT), which was recently reported in another unrelated *ADA*$^-$ immunodeficient patient[12], and has been observed in two additional unrelated *ADA*$^-$ SCIDs (R.H. *et al.*, unpublished data). We demonstrate here that both mutations were inherited; the IVS5+1 GT to AT mutation from his father and the Arg156His from his mother. We further demonstrate that the unexpected basis for somatic mosaicism was an *in vivo* spontaneous reversion to normal of the maternally transmitted mutation.

Residual ADA activity and metabolites

Adenosine deaminase (ADA) activity was not detectable in erythrocytes (RBCs) at age 5 but concentrations of deoxy ATP in RBCs and deoxyadenosine in urine were only minimally elevated, as compared to concentrations found in patients with early onset *ADA*⁻ SCID (Tables 1 and 2). Both parents exhibited approximately 50% of normal erythrocyte ADA as did two young adult healthy sibs. Enzyme activity in lymphocytes was diminished to approximately 15% of normal in the proband and 20–25% of normal (within the heterozygote range) in both the father and mother. Lymphoid cell lines established from the proband and both parents also exhibited markedly diminished ADA. Surprisingly however, enzyme activity in the cell line from the proband was indistinguishable from that in the parental cell lines. The considerable residual enzyme activity in non-erythroid cells and low concentrations of metabolites were similar to findings in 'partially' ADA deficient children ascertained by population screening who have remained healthy during the first years of life (ref. 13, references therein and unpublished data).

By contrast, the death in infancy due to immunodeficiency of a prior sib and the abnormal immunologic findings in the proband during the first years of life were more consistent with complete ADA deficiency (ref. 10 and case history). To clarify the basis for the apparent dichotomy, we undertook a molecular analysis of this family.

Table 1 Enzyme activity in uncultured and cultured cells

	RBCs	ADA[a] Lymphs	EBV LL	PNP[b] RBCs
Proband	<0.5	192.0	2, 040.0	40
Father	22.0	282.0	1, 951.0	34
Mother	35.0	306.0	1, 470.0	37
Sib G[c]	32.0	–	–	43
Sib C[c]	40.0	–	–	45
Normals	83.8 (58–134)	1,190.0 ± 128	4,100.0 ± 348	40 ± 5
ADA⁻SCIDs[d]	<0.5	9.4 (0.4–25)	9.7 (0.4–22)	

[a]nmoles/mg haemoglobin/h (± 2 SD log) or nmoles/mg protein/h (± SEM)
ADA activity for PB lymphocytes (Lymphs) represents activity inhibitable by erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), measuring only ADA encoded by the relevant chromosome 20 locus and excluding activity of a non-relevant isozyme that can represent 1–2% of normal ADA activity.
[b]PNP, purine nucleoside phophorylase activity; nmoles/mg haemoglobin/min
[c]Heterozygous for the Arg156His mutation
[d]Neonatal and delayed onset (n = 8)

Table 2 Metabolites in proband vs ADA deficient individuals of different phenotypes

		dATP[a] RBCs	(range)	dAr[b] urine	(range)
Proband		22		22.0	
'Partial'[c]	(n = 11)	16	(4–38)	6.0	(2–13)
CID[d]	(n = 2)	210	(174–247)	170.0	(54–270)
SCID[e]	(n = 9)	1,160	(327–2, 248)	1,033.0	(442–2,500
Normals	(n = 38)	4	(1–14)	<0.2	<0.2

[a]deoxy ATP; nmoles/ml packed RBCs (mother and father respectively 1.8 and 2.0.)
[b]deoxyadenosine; nmoles/mg creatinine
[c]Ascertained by screening norma50 healthy newborns
[d]Cellular immunodeficiency; late onset immunodeficiency
[e]SCID; neonatal and delayed onset immunodeficiency

A paternally transmitted mutation (IVS5 +1G-A)

Analysis of cDNA clones from the proband's lymphoid cell line revealed deletion of the 116-nt sequence contained in exon 5, with seven of the 19 cDNA clones smaller than normal size. A splice site mutation at the invariant G of the 5' donor site in IVS 5 (IVS5 +1GT to AT transition) was identified as the molecular basis for deletion of exon 5 from cDNA by sequence analysis of the proband's lymphoid line and peripheral blood (PB) genomic DNA. The splice site mutation was present in the father's genomic DNA from both lymphoid line and peripheral blood, as detected by loss of a *Bsr*I site (Fig. 1*a*) and confirmed by sequence analysis (data not shown).

Absence of maternal mutation in B cell line

To identify the maternally transmitted mutation, we examined the 12/19 additional cDNA clones that were of grossly normal size and which presumably represented the maternal allele. Two clones contained a G to A transition at nt 467, predicting an Arg156His substitution — a deleterious mutation previously reported in ADA deficient immunodeficient patients[11]. (As a probably incidental finding, one of these two clones also showed misplicing from mid exon 7 to exon 8 with a deletion of 27 bp in exon 7, a splicing error we have

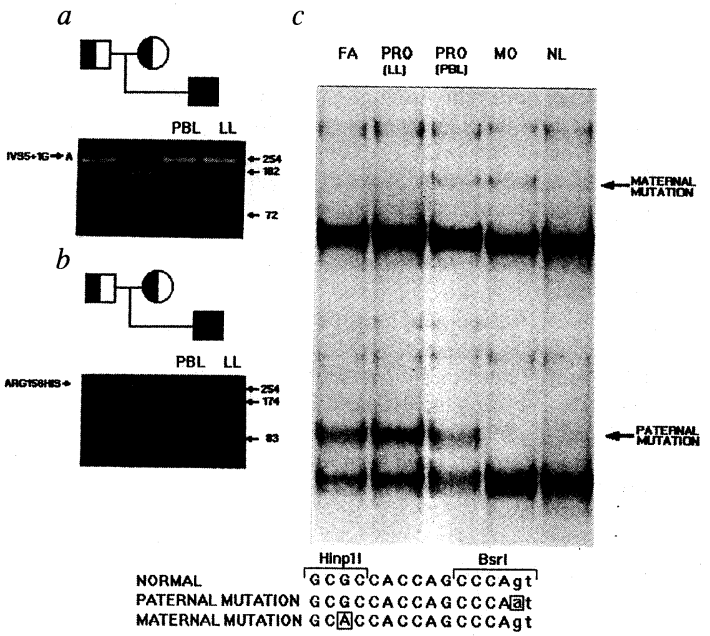

Fig. 1 *a*, Detection of a paternally transmitted splice site mutation (IVS5 +1 GT to AT) by loss of a *Bsr*I site A 254-bp fragment containg exon 5 was amplified from genomic DNA. The mutation abolishes the only *Bsr*I site, resulting in a 254-bp fragment in the proband and father. Identical results were obtained with PB and lymphoid line DNA. *b*, Detection of a maternally transmitted missense mutation (G467A, Arg156His) by loss of a *Hinp*1I site. The Arg156His mutation abolishes the only *Hinp*1I site in the 254-bp fragment amplified, resulting in the mutant 254-bp fragment. The absence of the mutation in the proband's lymphoid line but presence in his peripheral blood, as well as in both lymphoid line and PB DNA of the mother was confirmed by sequence analysis (data not shown). A faint 254-bp band in the proband's lymphoid line was shown to contain the mutation by purification and sequence analysis (data not shown). *c*, Single strand conformational polymorphism (SSCP) analysis of lymphoid line (LL) and peripheral blood (PBL) DNA consistent with absence of maternal mutation in proband's lymphoid line and presence in his PB DNA. Arrows indicate abnormal bands due to maternal and paternal mutations. FA= father, MO= mother, PRO= proband, NL= normal.

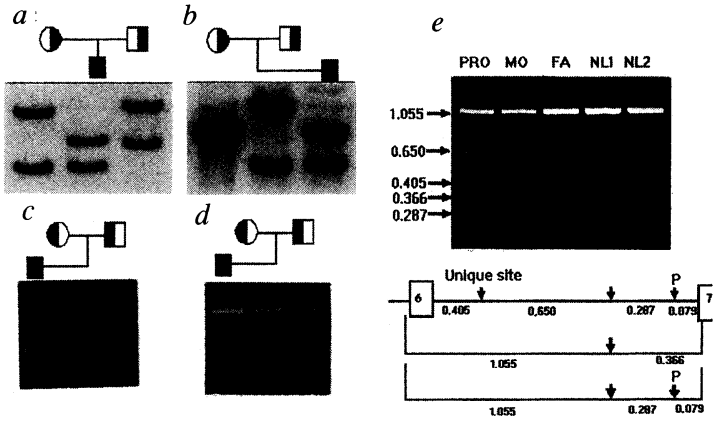

Fig. 2 Authenticity of paternal, maternal and child's lymphoid line cells. *a, b,* Analysis of VNTRs for *D20S19* (*a*) and *D16S85* (*b*) by hybridization of Southern blots. Alleles carried in the child's lymphoid line are consistent with inheritance from the mother and father. The results eliminate cross contamination and mislabelling between proband and paternal cell lines. *c, d,* Analysis of *ADA* RFLPs in lymphoid cell lines by PCR. *Pst*I *ADA* RFLP in IVS 2 (*c*) and *Bal*I (*Msc*I) *ADA* RFLP in exon 6 (*d*). The mother and father are both heterozygous for the IVS 2 and exon 6 ADA RFLPs and the child is homozygous for the more frequent allele (refs 23, 24). Identical results were found for PB DNA by PCR (data not shown). The results confirm the authenticity of the cell lines and eliminate the possibility of a mixup between the paternal and child's lymphoid line. Fuller analysis of *ADA* RFLPs by Southern blotting (data not shown) demonstrated that both mother and father carried I/III haplotypes but that the mother carried a unique *Msp*I site in IVS 6 on one allele. The child was homozygous for haplotype I/I, and heterozygous for the maternal 'private' *Msp*I RFLP (see (*e*)). (Haplotype I = common allele at all 7 sites, Haplotype III = rare allele at all sites except at an IVS3 *Msp*I RFLP[24]). *e,* Demonstration of a 'private' *ADA* IVS 6 *Msp*I RFLP. In addition to the 'private' *Msp*I RFLP in the mother and proband, a published *Msp*I RFLP at the 3' end of IVS6 (ref. 24) is seen, as illustrated in the diagram (arrows indicate *Msp*I sites, P = normal polymorphic site, unique site = *Msp*I site resulting in 'private' RFLP). The amplified fragment is cut by *Msp*I into a constant fragment of 1.055 kb plus either 0.366 or 0.287 + 0.79-kb fragments in homozygotes and all three fragments in heterozygotes for the reported IVS6 *ADA Msp*I RFLP (the 0.079-kb band is not visible here). The mother (MO), father (FA) and the second normal (NL 2) are heterozygous for this RFLP (0.366 + 0.287). The proband and the first normal (NL 1) are homozygous for the smaller allele (0.287). More significantly, the mother and child show 2 extra bands (estimated as 0.405 + 0.650 kb) produced by an additional *Msp*I site in the normally constant 1.055-kb fragment of one allele. Identical results were obtained by PCR of PB DNA from the child and parents (data not shown), further establishing authenticity of the child's lymphoid cell line lacking a maternal mutation.

seen only once before in cDNA carrying the adjacent C466T mutation[14], in multiple cDNAs sequenced.) All the remaining clones contained the normal G at nt 467, two of which were sequenced completely and contained totally normal sequence. The missense mutation was present in genomic DNA of the maternal lymphoid line and peripheral blood, as detected by loss of a *Hin*p1I site in exon 5 (Fig. 1*b*) and confirmed by sequence analysis (data not shown). Although the G467A (Arg156His) mutation was also clearly present in PB DNA from the proband, both by enzyme digestion and sequence analysis, the mutation was virtually absent in the proband's lymphoid line DNA (Fig. 1*b*). The trace presence of the G467A (Arg156His mutation) in DNA from the proband's lymphoid cell line was confirmed by purification of a faint band not digested by *Hin*p1I and sequence analysis (data not shown). SSCP analysis was also consistent with presence of the paternally transmitted splice site mutation in both lymphoid line and PB cells of the patient but absence of the maternally transmitted mutation in the lymphoid cell line despite presence in PB cells (Fig. 1*c*). The maternal Arg156His mutation was also found in the two healthy sibs who had heterozygous red blood cell (RBC) ADA activity (Table 1).

Authentication of proband's B lymphoid cell line

The virtual absence of the maternally transmitted mutation in the proband's lymphoid cell line and its clear demonstration in PB DNA may be explained by an *in vivo* reversion of an inherited mutation to normal in lymphoid cells. The findings could also result from two trivial causes: a major contamination of the proband's lymphoid line with the paternal cell line or an *in vitro* chance reversion to normal of the maternally transmitted mutation with selective growth in tissue culture of revertant cells. We first established the authenticity of the proband's cultured lymphoid cell line by comparison of DNA from the maternal, paternal and proband's cell lines for polymorphic markers and presence of a Y chromosome. We examined the highly polymorphic *D20S19* VNTR on chromosome 20 with over 10 alleles, the *D16S85* VNTR on chromosome 16 with over 30 alleles and multiple intragenic *ADA* RFLPs that included a maternally transmitted *Msp*I RFLP in *ADA* IVS 6 that is unique to this family (Fig. 2 *a–e*). Most significantly, the mother and child were both heterozygous for an additional site for *Msp*I in IVS 6, resulting in two additional restriction fragments (Fig. 2*e*). This maternally transmitted *Msp*I RFLP appears to be 'private' since we have not found this RFLP in over 100 chromosomes from patients or normals. Analysis of PB DNA from the proband and his parents for the *ADA* IVS 2 and exon 6 RFLPs as well as the private RFLP in *ADA* IVS 6 gave results identical to those for the respective lymphoid cell lines. Moreover, the two sibs who had inherited the maternal mutation also exhibited the maternally derived private *ADA* IVS6 *Msp*I RFLP. The combined data establish the authenticity of the proband's cell line lacking the maternally transmitted mutation and also linkage of the maternally transmitted Arg156His mutation in exon 5 and the ADA IVS 6 'private' RFLP.

Absence of mutations in some PB alleles

To determine if the reversion to normal of the maternally transmitted mutation had occurred *in vivo*, we initially analysed 89 clones, each containing a single allele, generated by PCR of PB DNA from two separate patient blood samples (Table 3). As expected, approximately 50% of clones carried the paternal mutation, detected by loss of a *Bsr*I site. By contrast, only 34% carried the maternal mutation, detected by loss of a *Hin*p1I site. Most significantly, 17% of clones did not carry either mutation. None of the clones carried both mutations. Absence of both mutations was confirmed

Table 3 Reversion to normal in individual alleles cloned from proband PB DNA: absence of both parental mutations

	Expt 1		Expt 2		Combined	
	n	%	*n*	%	*n*	%
Total clones	52	100	37	100	89	100
Paternal mutation	26	50	18	49	44	50
Maternal mutation	15	29	15	40	30	34
Neither mutation	11	21	04	11	15	17

see Methods for differences between experiments 1 and 2

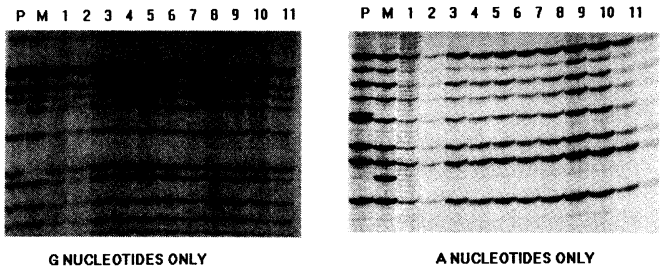

Fig. 3 Sequence analysis of individual cloned alleles lacking both maternal and paternal mutations. Left hand panel = sequencing for G only; right hand panel = sequencing for A only. Lane P = clone carrying paternal mutation (upper arrow), lane M = clone carrying maternal mutation (lower arrow), lanes 1–11 = clones lacking both mutations (Expt. 1, Table 3). Both the paternal and maternal mutations are G to A substitutions and therefore presence of the mutation is shown by loss of a normal G in the left panel and appearance of an A in the right panel. The four clones (from Expt. 2, Table 3) lacking a mutation by restriction digest were sequenced conventionally using all 4 deoxynucleotides, also confirming absence of both mutations (data not shown).

by single lane sequence analysis (Fig. 3) or complete sequence analysis (not shown). All 11 clones lacking a mutation and containing exons 5 and 6 carried the common allele for the *Msc*I RFLP in exon 6, consistent with heterozygosity of the parents and homozygosity of the child for this RFLP in PBL and lymphoid line DNA (Fig. 1*b*). To determine whether the reversion had occurred on the maternally or paternally inherited chromosome, we analysed clones containing IVS 6 for the 'private' *Msp*I RFLP carried by the mother and child. Clones carrying the maternally derived mutation carried this private RFLP in *ADA* IVS6, as did all four clones analysed that lacked both mutations (Fig. 4). Therefore these single allele clones that lacked both mutations must have resulted from reversion to normal of the maternal mutation.

Absence of maternal mutation in new B cell lines

To confirm *in vivo* reversion to normal of a transmitted mutation, we newly established multiple EBV transformed B cell clones and T cell pools from a blood sample obtained from the proband at age 11. Mutation analysis of 15 independent B cell lines revealed presence of the paternally derived mutation in all 15 but absence of the maternal mutation in 13/15 (Table 4). The maternal mutation was detected in two B cell lines, but in only trace amounts, as judged by the relative intensities of the undigested (maternal mutation)/digested *Hin*p1I bands. All 23 T cell pools contained both mutations, although the relative intensity of the undigested band suggested that there was a subpopulation of cells lacking the maternal mutation. B cell lines, whether lacking or containing the maternal mutation, as well as T cell pools, showed the maternally derived 'private' RFLP that is linked to the maternal mutation.

Discussion

Several observations forced us to test the unusual hypothesis that *in vivo* reversion to normal of an inherited mutation in a human gene had resulted in somatic mosaicism. These findings were: 1) the virtual absence of a maternally transmitted mutation in the child's original lymphoid cell line despite its presence in PB DNA; 2) the clear authenticity of this cell line; 3) the considerable residual ADA and low metabolite concentrations; and 4) the progressively milder clinical course in contrast to the early demise of a previously affected sib. By two separate approaches we have demonstrated *in vivo* reversion to normal of the maternally transmitted missense mutation (CGC467CAC; Arg156His). PB DNA contained individual alleles that did not carry a mutation and that were shown (by presence of a 'private' RFLP linked to the maternal mutation) to derive from the maternally transmitted allele. Secondly, 13 of 15 newly established B lymphoid cell lines totally lacked the maternal mutation, although this mutation was present in multiple T cell pools. Both B cell lines and T cell pools carried the maternally transmitted private RFLP, as well as the paternal mutation.

The relatively high residual ADA enzyme activity in lymphoid cells, low concentrations of metabolites and survival without therapy to the current age of 12 would appear to be primarily due to the demonstrated back mutation and resulting somatic mosaicism, rather than to possible 'leakiness' of the splice site mutation and/or expression of considerable residual ADA by the Arg156His mutation. While this work was in progress, both the IVS5 +1G–A and the Arg156His mutations were reported in several additional unrelated *ADA*$^-$ immunodeficient patients (heteroallelic with several different mutations). All of these other *ADA*$^-$ immunodeficient patients had less than 1% of normal enzyme activity in lymphoid cells and accumulated ten fold greater deoxy ATP than the child studied here[11,12]. Additionally, the deleterious nature of the Arg156His

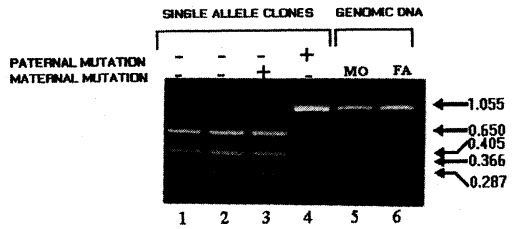

Fig. 4 Presence of a unique maternally transmitted RFLP in proband's cloned individual alleles either carrying the maternal mutation or lacking a mutation and absence in clones carrying the paternal mutation. (see also Fig. 2*e* for explanation of fragments) DNA from cloned individual alleles either carrying the paternal mutation, carrying the maternal mutation (Expt. 2, Table 3) or carrying neither mutation and, for comparison, PB genomic DNA of the mother (MO) and father (FA), was amplified, digested with *MSPI* and electrophoresed in 3% agarose gel. The single allele clone carrying the paternal mutation exhibits the common 1.055-kb and 0.287-kb *Msp*I fragments (lane 4). The single allele clones carrying the maternal mutation (lane 3) and all clones lacking a mutation all carry the unique 0.405 + 0.650 *Msp*I fragments in addition to the common normal 0.287-kb fragment (lanes 1 & 2).

Table 4 Mutation analysis of multiple newly established EBV B lymphoid cell lines and T cell pools

Mutation	T cell pools	EBV B cell lines
(+) paternal	23	15
(–) paternal	0	0
(+) maternal	23	2
(–) maternal	0	13

missense mutation was confirmed by expression studies[11]. In confirmation of these prior results, we have also directly compared the expression of 18 different ADA mutations, including the Arg156His mutation by the more sensitive method of transient expression and *in situ* histochemistry (manuscript in preparation). The Arg156His mutation expressed activity less than 1–2% of normal, similar to what we found for several other deleterious mutations and considerably less than that of any of the 'partial' ADA mutations. Therefore, based on findings in other patients carrying the same mutations, as well as *in vitro* expression studies, 'leakiness' cannot explain the 15% of normal ADA activity in the proband's lymphoid cells.

The mechanism for this site-specific reversion to normal of an inherited point mutation is undefined. Gene conversion appears unlikely in this case since one end of the conversion event would have to be within the 12 nt separating the sites of the maternal and paternal mutations. Apparent reversion to normal of mutations on one allele has been reported in regenerating liver nodules in four patients with the autosomal recessive disorder, tyrosinaemia[15]. Formal demonstration that the mutations was transmitted from the parents of the patients was not provided. However, it would seem unlikely that all four patients were mosaic for *de novo* mutations, supporting the hypothesis of back mutations. Two of these patients were homozygous for the mutation in non-regenerating liver, clearly excluding gene conversion. ADA deficiency and tyrosinaemia share several features of possible significance. Both disorders have increased concentrations of metabolites with potential to cause DNA damage, the alkylating agents fumaryl and maleyl-acetoacetate in tyrosinaemia and deoxyadenosine, which has been reported to induce DNA strand breaks *in vitro*, in ADA deficiency[15–17]. In both disorders, the abnormal metabolites also provide an *in vivo* selection system, potentially conferring a selective advantage on cells that have reverted one allele to normal. Lastly, both involve rapidly dividing cells (regenerating liver and lymphocytes). Since an increased mutation rate has not been actually demonstrated, a selective advantage alone could have allowed for selective survival and proliferation of sufficient spontaneous revertants derived from progenitor cell(s) to result in a detectable somatic mosaicism.

Our finding that a patient with ADA deficiency had site-specific reversion to normal of an inherited mutation may have important implications for evaluation and/or design of current trials of gene therapy that are combined with enzyme therapy. Such enzyme therapy might well tend to abolish the selective advantage and expansion of recombinant cells containing a normal allele, a hypothesis supported by a recent report of relative expansion of transduced cells carrying recombinant retroviral ADA following reduction in the amount of enzyme therapy[18].

Significant somatic mosaicism due to reversion to normal could be present in any disorder where normal cells have a selective advantage. The most obvious examples of a demonstrated selective advantage are the several X-linked immunodeficiency disorders where female carriers show selective survival of cells with the normal X chromosome active in the cell types affected by the disease (for example ref. 19). An additional candidate is X-linked HGPRT deficiency, where similar selective advantage is seen in haematopoietic cells of carrier mothers[20]. Differences in phenotype between sibs carrying single gene defects may therefore result not only from environmental differences or modifying cis or trans elements but also to chance reversion to normal of inherited mutation, selection and resulting somatic mosaicism.

Methods

Case history. The case history of the child used in our study to age 5 has been reported[10]. A prior sib had presented at 14 months with pneumonitis, failure to thrive, diarrhea and marked lymphopaenia affecting both T and B cells (2% lymphocytes with 8% T cells and 5% B cells). He died before three years of age with a diagnosis of 'Nezelof syndrome', now recognized as a variant of SCID with retention of some humoral immune function. The child studied here presented during the first 5 years of life with short stature, recurrent cutaneous staphylococcal abscesses, sinopulmonary infections, mucocutaneous candida infections, eosinophilia, marked hyper IgE and lymphopaenia, particularly of CD4 lymphocytes. At age 5 years he was found to lack ADA in erythrocytes but to exhibit 15% of normal ADA in mononuclear cells (see Table 1). Between the ages of 8 and 12 years, he has been clinically healthy, with normal growth and development despite the persistent findings of hyperimmunoglobulinaemia E, decreased absolute numbers of CD4 lymphocytes and B lymphocytes (CD19), and increased absolute numbers of CD8 lymphocytes and, on the most recent testing, the appearance of somewhat diminished *in vitro* lymphocyte response to mitogens. For religious reasons the child has not received transfusion of blood products, including gammaglobulin, PEG-ADA enzyme therapy, bone marrow transplants, nor undergone skin biopsy for establishment of fibroblasts. The parents are not consanguineous and 2 older male sibs are healthy.

Biochemical methods. Adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) activity and metabolite concentrations were determined as described[6,21,22].

Genotyping and cDNA analysis. Southern analysis of lymphoid line DNA for VNTRs, Y chromosome and PCR of PB and lymphoid line genomic DNA for *ADA* intragenic RFLPs were performed as described (refs 9, 23 and 24, and refs therein). mRNA was extracted and cDNA libraries generated both by classic methods and by RT/PCR using ADA specific primers as described[7,25]. Sequence analysis used either Sequenase (US Biochemical) or cycle sequencing with a fMole kit (Promega) as described[23].

Cloning and analysis of individual alleles from PB. Genomic DNA from two independent samples of PB from the proband was amplified by PCR and cloned into the TA vector pCRII (Invitrogen). DNA was extracted from PB leukocytes obtained at age 10 y (Table 3, expt 1), or from PB mononuclear cells sep-

arated by standard Hypaque-Ficoll purification from blood drawn at age 11 years (Table 3, expt 2). A 1.65-kb genomic DNA fragment containing exons 5 and 6 was amplified by PCR from sample 1 (Table 3, expt 1) with primers BG5299 (5'–CAGTCCCAGAGCTGCCCTTTAGGCCTTC–3') × BG3418 (ref. 23). A 2.7-kb fragment containing exons 5–7 was amplified from sample 2 (Table 3, expt 2), using primers BG5299 × BG4545 (5'–GTGGACAGTATGGTGAATGC-CGCTCT–3'). For analysis of mutations by restriction enzyme digest, a 254-bp fragment containing exon 5 was amplified from the clones using primers BG5299 × BG5298 (5'–CACT-GCTAGGCCAGGAGGTCAGGGC–3'). Exon 6 (ref. 23) or a 1.42-kb fragment containing exon 6–7 were amplified from the clones using primers BG3419 (ref. 23) × BG4545 for analysis of respectively, the *BalI* (*MscI*) RFLP in exon 6 or a 'private' *MspI* RFLP in IVS 6, unique to mother and child[23,24]. A 1.421-kb fragment extending from just 5' of exon 6 to the beginning of exon 7 was amplified from lymphoid lines of the proband (PRO), mother (MO), father (FA) and PB of two normals (NL 1 and NL 2), digested with *MspI* and electrophoresed in 3% agarose. The order and size of fragments for the 'private' RFLP were determined by double enzyme digests as well as analysis of Southern blots hybridised with an exon 6 specific genomic probe which also demonstrates the 'private' RFLP.

Detection of mutations in genomic DNA. Genomic DNA from EBV transformed B cell lines and T cell pools or PB DNA was amplified using nested PCR of exon 5 (primers BG5151 × DG747 (ref. 9) followed by BG5299 × 5298). Individual cloned alleles derived by PCR of PB DNA were tested by enzyme digestion for presence of the paternal and maternal mutation and clones lacking both mutations were then sequenced. SSCP was performed and analysed on MDE gels as described by the manufacturer (J.T. Baker), based on described methods[26].

Cell lines. The original EBV transformed cell lines from the patient and his parents were established when the patient was 5 years old, at the time of enzyme and metabolic studies. These cell lines (GM 10077, 10078 and 10079) are deposited with the NIH Mutant Cell Repository in Camden, NJ. Multiple EBV transformed B cell lines and T cell pools were newly established from the blood sample obtained at age 11 years. Fifteen independent EBV B cell lines were generated in microtiter plates by standard methods [27] and expanded for 3–5 weeks for analysis of DNA. Purified activated T lymphocyte cultures were prepared as described by stimulation with PHA for 3 days followed by expansion of non-adherent cells for 2–4 weeks in the presence of recombinant human IL-2 (ref. 28).

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