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Splicing Defects in the Ataxia-Telangiectasia Gene, *ATM:* Underlying Mutations and Consequences

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Summary

Mutations resulting in defective splicing constitute a significant proportion (30/62 [48%]) of a new series of mutations in the *ATM* gene in patients with ataxia-telangiectasia (AT) that were detected by the protein-truncation assay followed by sequence analysis of genomic DNA. Fewer than half of the splicing mutations involved the canonical AG splice-acceptor site or GT splice-donor site. A higher percentage of mutations occurred at less stringently conserved sites, including silent mutations of the last nucleotide of exons, mutations in nucleotides other than the conserved AG and GT in the consensus splice sites, and creation of splice-acceptor or splice-donor sites in either introns or exons. These splicing mutations led to a variety of consequences, including exon skipping and, to a lesser degree, intron retention, activation of cryptic splice sites, or creation of new splice sites. In addition, 5 of 12 nonsense mutations and 1 missense mutation were associated with deletion in the cDNA of the exons in which the mutations occurred. No ATM protein was detected by western blotting in any AT cell line in which splicing mutations were identified. Several cases of exon skipping in both normal controls and patients for whom no underlying defect could be found in genomic DNA were also observed, suggesting caution in the interpretation of exon deletions observed in *ATM* cDNA when there is no accompanying identification of genomic mutations.

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Introduction

Ataxia-telangiectasia (AT [MIM 208900]) is an autosomal recessive disorder with a diverse phenotype that includes progressive cerebellar ataxia, oculocutaneous telangiectasias, radiation hypersensitivity, increased cancer incidence, immunodeficiency, chromosome instability, and elevated levels of serum alpha-fetoprotein (reviewed in Gatti 1991, 1998). Genetic studies of families with AT indicate that heterozygous carriers for AT, although generally asymptomatic, have an increased risk for the development of breast cancer (Swift et al. 1991; Athma et al. 1996). The gene mutated in AT, *ATM*, was identified in 1995 (Savitsky et al. 1995*a*, 1995*b*; Uziel et al. 1996). In light of both the public-health implications of the reported cancer risk in known AT carriers and estimates that place their population frequency at 0.5%–1%, there is substantial interest in detection and characterization of mutations in *ATM* (Swift et al. 1991; Easton 1994; FitzGerald et al. 1996; Vorechovsky et al. 1996).

The majority of mutations detected thus far in the *ATM* gene in patients with AT are predicted to result in protein truncation (Gilad et al. 1996; Concannon and Gatti 1997). Western blot analyses of cell lines of patients with AT, with antibodies directed against ATM, indicate that most of these truncating mutations are associated with an absence of detectable ATM protein (Lakin et al. 1996; Stankovic et al. 1998). Some studies have reported that patients with AT whose cells are capable of producing even modest amounts of ATM may have distinct phenotypic features when compared with those whose cells produce no detectable protein. One class of mutations that has the potential to be "leaky" and to allow for the production of at least some ATM protein are those that affect the fidelity of splicing. Indeed, two such mutations with effects on splicing of *ATM* have been reported as being associated with either a milder phenotype (McConville et al. 1996) or a variant

Table 1

ATM Primers

Primers Used	Nucleotides	Designation
For amplification of cDNA:		
CAGAAGAGCACCTAGGCTAA	327–346	14F
CTGCCGTTATAATGCTTTAGG	1023–1003	14R
CGAGTGTGTGAATTAGGAGAT	748–778	AF3
CATTAAGCCTATGAAGAG	817–800	AR2
TGTTCTCTGTTTACTTCAG	500–685	ex8.2F
AGAATGATTTTGATCTTGTGC	1231–1211	11AR
GCTTACTTGGAGCCATAATTC	1517–1537	16F
GTGAACACCGGACAAGAGTT	2158–2139	16R
CGCTGTCTTCTGGGATTATC	2124–2093	17F
GTAGGTTCTAGCGTGCTAGA	2816–2797	17R
GTGTAACTACTGCTCAGACC	2720–2739	18F
GTTTCAGGGTTCTCAGCACT	3428–3409	18R
TGCATACTTGAAAGCTCAGGA	3366–3386	19F
TCAGTGCTCTGACTGGCACT	4091–4072	19R
AGACAGCCGTGACTTACTGTA	4508–4528	3F
CAGGATTATGAAGGTCCACTG	5145–5115	3R
TTGTCTTCGAAGATCC	4860–4845	R5
CAGTGGAGGCACAAAATGTGA	5445–5466	5F
CTGGCTTCCTTCTTCAAATGC	5948–5926	5R
CTCTATGCAGAAATCTATGCAG	5866–5887	6F
CTGGTTCCTTCTACTTCTTTGC	6368–6347	6R
CACTGCATATTCCTCCATGCTGC	6316–6304	R309
CAACCTGATTGTGTGGGATAG	6528–6507	B2
T7–ATGCAGTGGGACCATTGC	6319–6335	7F
GAGACTCCACAGCTAACTGAA	6866–6846	7R
GAAGTAGGTCTCCTTAGGGAA	7267–7287	9F
TTCTGACCATCTGAGGTCTCC	7855–7834	9R
TTGATGAGGATCGAACAGAGG	7780–7801	10F
CATTCAAGAACACCACTTCGC	8306–8285	10R
CACGGAAACTAGGAAGAGGAA	8226–8246	11F
CCCTGGTTTTCTCACAGCAT	8806–8787	11R
ATGAGCAGTCAGCAGAACTTG	8636–8656	12F
CTAAAGGCTGAATGAAAGGGTAAT	9209–9185	12R
	Exon	Product Size (bp)
For amplification of genomic DNA:		
CCCCCTGTTATACCCAGTT	9F	318
TGAAGAAGCAAATTCAAAACAG	9R	
TTTGTGGGGAGCTAGCAGTG	10F	262
TCTAAATGTGACATGACCTAC	10R	
GTTTGTTAATGTGATGGAATA	12F	467
GTGTGTTTATCTGTAAGTCAG	12R	
GTTCTTACAAAAGATAGAGTATAC	16.2F	329
TTCACAGGAATACATTTCATTC	16.2R	
GTCCAAGATCAAAGTACACTG	17.2F	314
GTGACAGAGAAAGATCCTATC	17.2R	
ATATTGGCCCTAATAGTAAAC	18F	292
CCTTATTTACAAAGATATTTCAAC	18.2R	
CCGGCCTATGTTTATATACTT	21F	225
TTAACAGAACACATCAGTTAT	21R	
TGGAGTTCAGTTGGGATTTTA	26F	304
TTCACAGTGACCTAAGGAAGC	26R	
TGCTGAACCAAAGGACTTCT	32F	334
CACTCAAATCCTTCTAACAATA	32R	
GTATGTTGAGTTTATGGCAGA	39F	376
ATCCATCTTTCTCTAGAACTG	39R	
ACCAGAACCTTATAGCATAGT	40F	247

(*continued*)

Table 1 (continued)

Primers Used	Nucleotides	Designation
	Exon	Product Size (bp)
TTCAGCCGATAGTTAACAAGT	40R	
TAAGCAGTCACTACCATTGTA	41F	314
ATACCCTTATTGAGACAATGC	41R	
GTATATGTATTCAGGAGCTTC	42F	238
ATGGCATCTGTACAGTGTCT	42R	
CAGAACTGTATTTCAGAATCAT	43F	387
ACATAACTCCTTCATAAACAGT	43R	
CCAAAGCTATTTTCACAATCTT	44F	262
TACTGAAATAACCTCAGCACT	44R	
CTCTGGTTTTCTGTTGATATC	45F	236
CCCCATGAAGAATCAAGTC	45R	
TTTATACATGTATATCTTAGGGTTCTG	46F	220
TTCAGAAAAGAAGCCATGACA	46R	
CACTGCAGTATCTAGACAGT	54F	322
CTAGGAAAGACTGAATATCAC	54R	
AATGTTGGGTAGTTCCTTATG	55F	308
GCTTTTGGATTACGTTTGTGA	55R	
CCTTTGCTATTCTCAGATGACTCTGT	58F	290
GCATTATGAATATGGGCATGA	58R	
TAGAAAGAGATGGAATCAGTG	61F	317
ATCTTGGTAGGCAAACAACAT	61R	
AAAGTTCACATTCTAACTGGAA	62F	272
ATTACAGGTGCAAAGAACCAT	62R	
TCCTGTTGTCAGTTTTTCAGA	65F	354
ACTTAAAGTATGTTGGCAGGT	65R	

[a] Sequence is from Vorechovsky et al. (1996).

clinical presentation (Gilad et al. 1998), even in compound heterozygotes.

There is suggestive evidence that splicing-related mutations may be unusually frequent in AT. A recent survey of published reports of *ATM* mutations included 115 alterations detected predominantly through the examination of *ATM* cDNA from patients with AT (Concannon and Gatti 1997; for updates, see the Ataxia-Telangiectasia Mutations Database Website). A significant fraction (45/115 [39.1%]) of these changes corresponded to the loss of either exons or portions of exons, presumably reflecting the presence of underlying splice-site mutations. Unfortunately, for the majority (39/45) of these *ATM* splicing alterations, only the observed effects in cDNA were reported, not the identity of specific causative mutations. If it is assumed that such mutations can be identified in the majority of cases, then the frequency of splicing mutations in the *ATM* gene would be substantially higher than that reported in surveys of other human genetic disorders, in which ~15% of point mutations are found to affect mRNA splicing (Krawczak et al. 1992; Ruttledge et al. 1996).

In the present study, we have sought confirmation of the implied high frequency of splicing mutations in *ATM*, through the study of an independent population of patients with AT. Initial mutation screening was done at the cDNA level, to identify any cell lines from patients with altered splicing patterns. In all such cases, the underlying mutation was then identified by sequencing of genomic DNA. To evaluate the possible implications that the different splicing mutations have for ATM expression, all cell lines of patients were tested for the production of ATM, by western blotting. Where appropriate, clinical features of specific patients were reexamined in light of the mutation data.

Patients, Material, and Methods

Patients

Genomic DNA and mRNA were isolated, and cDNA was synthesized, from B-lymphoblastoid cell lines previously established from patients with AT. Patients were ascertained in Turkey, Poland, Italy, and the United States. The clinical features for all patients were reviewed by one of us (R.A.G.) and were found to be consistent with the standard diagnostic criteria for AT. All clinical diagnoses were confirmed by radiation-sensitivity testing of cell lines by the colony-survival assay (Huo et al. 1994). Cell lines AT25RM, AT39RM, AT117LA, and

Table 2

Frequency of Mutations Associated with Splicing Defects in Patients with AT

AT Cell Line	Genomic Mutation[a]	Effect on cDNA	Intron/Exon	Codon Change	Codon Number[b]	Status	Consequence	Second Mutant Allele
AT68LA	380delA	380delA	7	Frameshift	128	Heterozygote	Truncation	Unknown
AT113LA	748C→T	663del239	9	R→X	221	Heterozygote	Exon 9 skipped, truncation	Unknown
AT51LA	802C→T	663del239	9	Q→X	268	Heterozygote	Exon 9 skipped, truncation	Unknown
AT134LA	IVS9−1G→T	902del164	10	Frameshift	301	Heterozygote	Exon 10 skipped, truncation	3802delG
AT139LA	1024delAAAG	1024delAAAG	10	Frameshift	343	Heterozygote	Truncation	Unknown
AT17LA	1180delGG	1180delGG	11	W→X	393	Heterozygote	Truncation	Unknown
AT81LA	1563delAG	1563delAG	12	Frameshift	522	Heterozygote	Truncation	6100C→T
AT115LA	1563delAG	1563delAG	12	Frameshift	522	Homozygote	Truncation	…
AT39LA	1563delAG	1563delAG	12	Frameshift	522	Heterozygote	Truncation	3382C→T
AT39RM	IVS12+1G→T	IVS12ins764	12	Frameshift	535	Homozygote	Intron 12 retained, truncation	…
AT54LA	2250G→A	2125del126	16	Silent	709	Heterozygote	Exon 16 skipped	8266A→T
AT111LA	IVS16−10T→G	IVS16−1ins9	17	Frameshift	750	Heterozygote	Splice acceptor created, truncation	2809insCTAG
AT114LA	IVS18+1G→A	2237del90	18	del30	792	Heterozygote	Exon 18 skipped	Unknown
AT25RM	2503insA	2503insA	19	Frameshift	835	Heterozygote	Truncation	Unknown
TAT50	2503insA	2503insA	19	Frameshift	835	Homozygote	Truncation	…
TAT49	IVS21+3insT	2839del83	21	Frameshift	946	Homozygote	Exon 21 skipped, truncation	…
AT84LA	3372C→G	3372C→G	25	Y→X	1124	Heterozygote	Truncation	Unknown
AT142LA	3576G→A	3403del174	26	Silent	1135	Heterozygote	Exon 26 skipped	Unknown
TAT48	3576G→A	3403del174	26	Silent	1135	Homozygote	Exon 26 skipped	…
AT117LA	3576G→A	3403del 174	26	Silent	1135	Heterozygote	Exon 26 skipped	Unknown
AT136LA	3625delTT	3625delTT	27	Frameshift	1209	Heterozygote	Truncation	Unknown
AT36LA	3663G→A	3663G→A	27	W→X	1221	Heterozygote	Truncation	1110delC
AT80LA	3802delG	3802delG	28	Frameshift	1268	Heterozygote	Truncation	Unknown
AT18RM	3894insT	3894insT	28	Frameshift	1299	Homozygote	Truncation	Unknown
AT137LA	4052delT	4052delT	29	Frameshift	1351	Heterozygote	Truncation	Unknown
AT140LA	4373delG	4373delG	31	Frameshift	1458	Heterozygote	Truncation	Unknown
AT11LA	IVS32−12A→G	IVS32−1ins11	32	Frameshift	1479	Homozygote	Splice acceptor created, truncation	…

AT46LA	5290delC	5290delC	37	Frameshift	1764	Heterozygote	Truncation	Unknown
AT32LA	IVS38−2A→C	5497del61	39	Frameshift	1832	Heterozygote	Cryptic splice acceptor, truncation	Unknown
TAT51	5623C→T	5623C→T	39	R→X	1875	Homozygote	Truncation	…
AT56LA	IVS40+1126A→G	IVS40ins137	40/41	Frameshift	1921	Heterozygote	Cryptic splice sites, truncation	Unknown
CAT13	IVS40+1126A→G	IVS40ins137	40/41	Frameshift	1921	Heterozygote	Cryptic splice sites, truncation	Unknown
AT130LA	5932G→T	5919del88, 5932G→T	42	R→X	1973	Homozygote	Exon 42 skipped, truncation	…
AT63LA	5932G→T	5919del88, 5932G→T	42	R→X	1973	Heterozygote	Exon 42 skipped, truncation	432insA
AT31LA	5971G→T	5919del88, 5971G→T	42	E→X	1991	Heterozygote	Exon 42 skipped, truncation	7636del9
AT65LA	6095G→A	6007del89	43	Frameshift	2003	Heterozygote	Exon 43 skipped, truncation	214G→T
AT138LA	6154G→A	6096del103	44	Frameshift	2032	Homozygote	Exon 44 skipped, truncation	…
AT127LA	IVS45+1G→A	6007del326, 6347insAG, 6347ins78nt	45	Frameshift	2002	Homozygote	Exon 43, 43–45 skipped, truncation	…
AT30LA	IVS46+1G→A	6348del105	46	del35	2176	Heterozygote	Exon 46 skipped	6372insG
GM01525C	6404insTT	6404insTT	46	Frameshift	2136	Heterozygote	Truncation	Unknown
AT72LA	IVS53−2A→C	7630del159	54	del53	2544	Heterozygote	Exon 54 skipped	3085insA
AATV26	7705delGA	7705del GA	54	D→X	2569	Heterozygote	Truncation	Unknown
AT119LA	7865C→T	7864del64	55	Frameshift	2621	Homozygote	Splice donor created, truncation	…
AT121LA	8264delATAAG	8152del117	58	del39	2718	Heterozygote	Exon 58 skipped	3485T→G
TAT45	IVS60−14del27	8585del87	61	del29	2886	Homozygote	Exon 61 skipped	…
AT147LA	IVS62+1G→A	8672del115	62	Frameshift	2891	Heterozygote	Exon 62 skipped, truncation	6998insA
AT122LA	IVS62+1G→A	8672del115	62	Frameshift	2891	Heterozygote	Exon 62 skipped, truncation	Unknown
AT73LA	8769insT	8769insT	62	Frameshift	2924	Heterozygote	Truncation	Unknown
AT34LA	IVS64−1G→C	8988del13	65	Frameshift	3045	Heterozygote	Cryptic splice acceptor, truncation	Unknown

NOTE.—Mutations associated with splicing defects are enclosed in boxes.

[a] Numbering of nucleotides is based on the sequence reported by Savitsky et al. (1995*b*), which designates the first nucleotide of the initiating ATG codon as "1"; mutations are designated according to the convention of Beaudet and Tsui (1993), as modified by Antonarakis (Recommendations for a Nomenclature System for Human Gene Mutations). Exon/intron boundaries and numbering are as defined by Uziel et al. (1996), with introns numbered such that intron X follows exon X, −2 and −1 are the positions of the splice acceptor AG, and +1 and +2 are the positions of the splice donor GT.

[b] In the case of a frameshift mutation, the codon given is that at which the predicted protein sequence diverges from the normal ATM protein sequence.

AT18RM were provided by one of us (L.C.). SV40-transformed fibroblast cell lines LM217 and GM00637, from two individuals with normal radiosensitivity, were obtained from Dr. L. Kapp (University of California, San Francisco). A B-lymphoblastoid cell line was established from unaffected individual NAT2.

Primers

Primers used for the protein-truncation test (PTT) assay have been described by Telatar et al. (1998). Primers used to amplify portions of the cDNA for SSCP and sequence analyses, as well as primers used to amplify exons from genomic DNA, are listed intable 1.

RNA Isolation, cDNA Synthesis, and the PTT

Specific methods for RNA isolation, cDNA synthesis, and application of the PTT to *ATM* have been described elsewhere (Telatar et al. 1996). Protein products from the coupled in vitro transcription-translation reaction were separated on 10%–20% gradient SDS-PAGE gels for 5 h at 250 V.

SSCP

SSCP analyses (Orita et al. 1989) were done on both cDNA and genomic DNA. PCR amplifications were done in 50-μl reactions containing 200 mM each of dCTP, dGTP, and dTTP (Boehringer Mannheim), 10 mM dATP (Boehringer Mannheim), 0.1–0.2 mCi of [^{33}P]-dATP (Amersham), 1 ng of each primer, 1.25 units of *Taq* DNA polymerase (either Ampli*Taq* from Perkin-Elmer/ABI or *Taq* polymerase from Boehringer Mannheim), and 50 ng of either cDNA or genomic DNA. The amplification profile was 35 cycles of 30 s at 94°C, 1 min at 55°C, and 1 min at 72°C, followed by a 7-min extension at 72°C. A 4-μl aliquot of the PCR product was mixed with 10 μl of dye buffer (98% formamide, 10 mM EDTA pH 8, 0.05% bromophenol blue, and 0.05% xylene cyanol) and was heated for 5 min at 95°C, quick-cooled to 4°C, and held on ice. Four microliters of each sample was loaded on both a standard 0.5 × MDE (mutation-detection enhancement [FMC]) gel and a 0.5 × MDE gel supplemented with 5% glycerol. After electrophoresis for 15 h at 7 W, gels were dried and exposed to x-ray film for 24–48 h. Variant or wild-type bands were cut from the gel, rehydrated in 20 μl of distilled water, reamplified by PCR, and sequenced.

Sequencing

PCR reactions for direct sequencing of 50 ng of genomic DNA were done in 50-μl volumes containing 200 mM each of dATP, dCTP, dGTP, and dTTP (Boehringer Mannheim), 0.3 mM of each primer, and 1.25 units of *Taq* DNA polymerase (Perkin-Elmer/ABI or Boehringer Mannheim) in PCR buffer. Amplification of cDNA for sequencing was done as described for genomic DNA, except that 1 ng of each primer was used. High Pure™ columns (Boehringer Mannheim) were used to purify the PCR products to be sequenced. Purified PCR products (1–10 μl) were sequenced with an FS sequencing kit (Perkin-Elmer/ABI), were purified with Centri-sep™ columns (Princeton Separations), and were electrophoresed on an ABI 373 automated sequencer.

Western Blotting

Epstein-Barr virus–transformed lymphoblastoid cells were grown in RPMI 1640 supplemented with 15% fetal bovine serum, 100 μg of penicillin/ml, and 100 μg of streptomycin/ml. Cell pellets of ~1 × 10^6 cells were washed twice with PBS (80 mM Na_2HPO_4, 15 mM KH_2PO_4, 1.4 M NaCl, and 27 mM KCl) pH 7.4 and then were lysed by sonication in 100 μl of cell lysis solution (Analytical Chemiluminescent Laboratories) containing 2 μg of aprotinin/ml and 550 μM phenylmethylsulfonylfluoride. During the procedure, the samples were kept on ice whenever possible. After sonication, the samples were centrifuged at 15,300*g* for 7 min at 4°C. The supernatants were recovered, and the protein concentration was measured, by a modified Bradford method (Bio-Rad). A total cell lysate of 25 μg was electrophoresed on 6% SDS-polyacrylamide gels and was blotted onto PVDF membranes (Bio-Rad) overnight at 4°C. The efficiency of the transfer was verified by silver-staining of the gel. Membranes were blocked with 10% nonfat milk for 2 h. ATM protein was detected by a monoclonal antibody generated against peptide 980–1512 in the leucine zipper and proline-rich region of the ATM protein (Chen and Lee 1996). The primary antibody was applied for 1 h. The membrane was then washed six times with PBS-Tween 0.05%. Peroxidase-linked anti-mouse Ig (Amersham) was applied for 30 min, and the membranes were washed six times with PBS-T. Detection was done by the ECL system (Amersham). Blotting of serial dilutions of lysates from normal control cell line NAT2 was used to establish conditions under which 10% of the normal level of ATM could be reliably detected.

Results

Alterations in *ATM* cDNA were detected by either SSCP or PTT in 49 cell lines derived from patients with AT. Within these cell lines, a total of 62 mutations, corresponding to 75/98 (76%) of the disease chromosomes, were identified in genomic DNA by both SSCP analysis and DNA sequencing of exons (table 2). Some of the cell lines are representative of founder-effect mutations in specific ethnic groups, as described elsewhere (Telatar et al. 1998). However, 32/62 (52%) of the mutations reported here are novel. Of the 62 mutations, 30 (48%)

affected the accuracy of splicing of the *ATM* transcript. In all cell lines in which mutations were identified, western blotting was used to determine whether any detectable normal-size ATM protein was produced.

Mutations in Canonical Splice Sites

Only 10 of the 30 *ATM* splicing mutations detected in the present study altered either a conserved GT or AG dinucleotide. Of the five mutations in the GT dinucleotide splice donor, three predictably resulted in skipping of the adjacent exon, whereas the remaining two mutations had unexpected effects (table 2).

In AT39RM, homozygous for the splice-donor substitution GT→TT in intron 12, two cDNA products were visualized by SSCP, as shown in figure 1*B* and as diagrammed in figure 2*B*. The upper band in lane AT39RM in figure 1*B* corresponds to an aberrantly spliced cDNA from which intron 12 has not been removed. A small amount of the correctly spliced cDNA is also seen (and confirmed by direct sequencing of the cDNA), despite the absence of an intact splice-donor sequence. Of the 30 splicing mutations detected in the present study, only AT39RM exhibited intron retention. Retention of intron 12 is predicted to lead to a frameshift and truncation of the protein. No ATM protein was detected by western blotting in AT39RM, indicating that, although some correctly spliced *ATM* transcript was produced in this cell line, it did not lead to the accumulation of detectable amounts of ATM protein (fig. 3).

In AT127LA, a homozygous mutation, GT→AT, of the splice-donor sequence at the end of exon 45 (IVS45+1G→A) resulted in the production of multiple incorrectly spliced transcripts identified in cDNA (see the numbered bands in fig. 1*C*, lane AT127LA). These transcripts corresponded to an insertion of 72 nucleotides from intron 45, an insertion of 80 nucleotides, an insertion of 2 nucleotides between exons 45 and 46, and a fourth species (data not shown) that had a deletion of exons 43–45. No normally spliced transcripts (shown in band 3a in fig. 1*C*, lanes N1 and N2) were detected in AT127LA, and, as expected, no ATM protein could be detected in cell lysates by western blotting (fig. 3).

The effects of mutations at the conserved AG dinucleotide of the splice acceptor were similarly diverse. Two mutations, IVS9-1G→T (in AT134LA) and IVS53-2A→C (in AT72LA), resulted in skipping of exons 10 and 54, respectively (table 2). However, two other similar mutations, IVS64-1G→C (in AT34LA) and IVS38-2A→C (in AT32LA), each resulted in the activation of cryptic splice sites (table 2).

Mutations at Positions Flanking Canonical Splice–Site Sequences

Three mutations were identified that altered nucleotides at the less-conserved positions (Padgett et al. 1986;

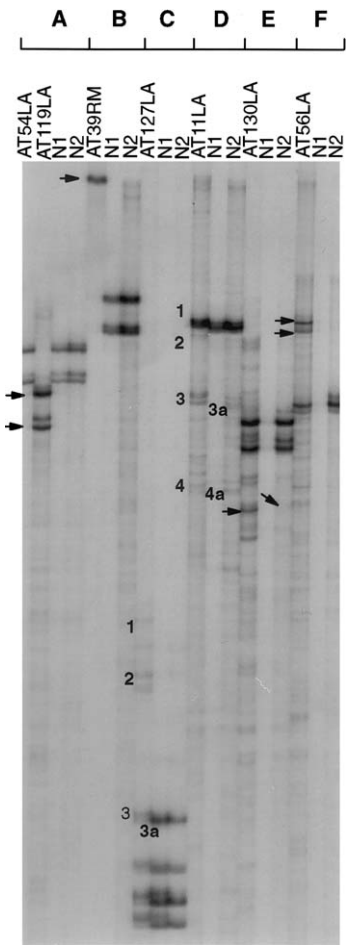

Figure 1 SSCP of *ATM* subfragments from patients with AT and from controls. In each of the panels *A–F*, cDNAs from AT cell lines and from controls GM637 (lane N1) and LM217 (lane N2) are compared. *A*, *ATM*7781-8305, including portions of exons 55–58. The arrows in lane AT119LA indicate the two strands of the mutant transcript deleting 64 nucleotides at the end of exon 55. *B*, *ATM*1517-2159, including portions of exons 12–16. The arrow in lane AT39RM indicates the mutant transcript in which intron 12 was not removed. *C*, *ATM*6319-6529, including portions of exons 45–47. In lane AT127LA, band 1 represents a 72-nucleotide insertion from intron 45, band 2 represents an 80-nucleotide insertion from intron 45, and bands in the region below band 3 all have AG inserted between exon 45 and exon 46. Bands in region 3a of the control lanes show the slightly shifted positions of the wild-type transcripts. *D*, *ATM*4508-5145, including portions of exons 32–36. In lane AT11LA, band 1 refers to the doublet representing the two strands of the mutant transcript in which 11 nucleotides of intron 32 were inserted. Bands near 3 and 4 represent transcripts that contained the 11 nucleotides of intron 32 and skipped either exon 34 or exons 34 and 35, respectively. Corresponding bands 3a and 4a of the control (lane N2) did not contain the intron 32 insertion but skipped exon 34 or exons 34 and 35, respectively. Band 2 of lane AT11LA contained the intron 32 insertion and had deletion of the first 25 nucleotides of exon 34. *E*, *ATM*5866-6368, including portions of exons 41–45. The arrow in lane AT130LA indicates a transcript lacking exon 42. The arrow in the control (lane N2) indicates a transcript that had deletion of the first 49 nucleotides of exon 45. *F*, *ATM*5445-5948, including exons 39–41. The arrows indicate the two strands of the mutant transcript in which 137 nucleotides from the middle of intron 41 were inserted.

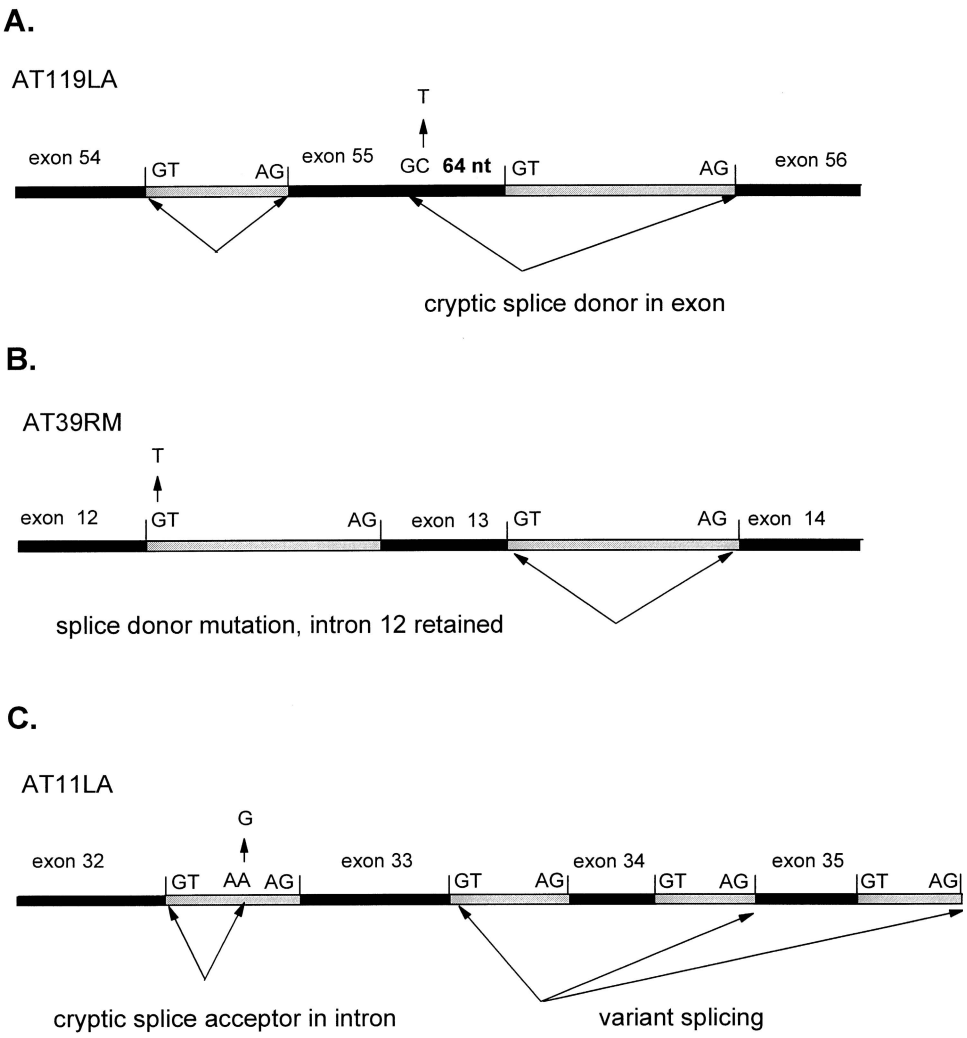

Figure 2 Effects of *ATM* splice mutations. *A,* Mutation 7865C→T, which creates a splice donor (GT) within exon 55, resulting in the deletion of 64 nucleotides at the end of the exon. Arrows indicate sites joined by splicing on this mutant allele. *B,* Mutation IVS12+1G→T of the splice donor in intron 12, which abolishes normal splicing of intron 12. Intron 13 is correctly spliced, as indicated by arrows. *C,* Mutation IVS 32-12A→G, which creates a splice acceptor (AG) in intron 32, resulting in the inclusion of 11 nucleotides of intron 32 in the transcript. No mutations were detected in the splice sites of exon 34 or exon 35, but either one or both of these exons were deleted in some of the transcripts from AT11LA, as indicated by arrows, suggesting that variant splicing occurred.

Krawczak et al. 1992) flanking the invariant GT dinucleotide of the splice donor. Two patients (TAT48 and AT117LA) had a 3576G→A mutation altering the last nucleotide of exon 26, and another patient (AT54LA) had an analogous mutation affecting the last nucleotide of exon 16 (2250G→A) (table 2). In all three cases, the nucleotide substitution itself is silent with respect to the amino acid sequence. Patient TAT48 is homozygous for the 3576G→A mutation and thus is particularly illustrative with regard to the effect that such a substitution has at the last nucleotide of this particular exon. The only transcript detected in this cell line had exon 26 deleted (fig. 4), as shown by SSCP and as confirmed by direct sequencing, indicating that the G→A substitution was sufficient to completely disrupt normal splicing. Deletion of exon 26 should result in a transcript that, if translated, would leave the reading frame of the ATM protein intact but that would eliminate 58 amino acids. Nevertheless, no ATM protein of normal or altered molecular weight was detected in patient TAT48 by western blotting (fig. 3).

The mutation IVS21+3insT in patient TAT49 highlights the significance of two other, less well-conserved positions in the consensus splice site. Patient TAT49 is homozygous for the insertion of T at the +3 position, leading to the altered splice-donor sequence TC | GTTAAGAA (position +3 is underlined, and the vertical bar denotes the intron/exon boundary), compared with

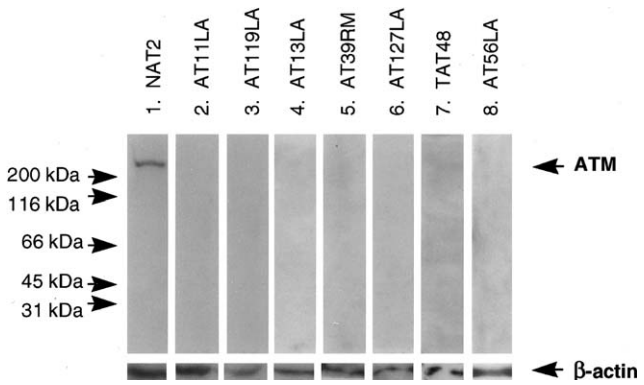

Figure 3 Western blots of proteins from cell lysates for seven patients with AT and for a control. ATM protein was detected with a monoclonal antibody generated against peptide 980-1512 in the leucine zipper and proline-rich region of the ATM protein (Chen and Lee 1996). Control NAT2 is represented in lane 1. β-Actin antibody provides an internal standard for the amount of protein per lane. Lanes 1–3, 5, and 6 were taken from one blot, whereas lanes 4, 7, and 8 represent separate experiments. In all cases, ATM was readily detected in the control.

the wild-type sequence TC | GTAAGAAA. The mutation in patient TAT49 disrupts the consensus sequence, placing the rarely used residue T (2%) at the +3 position and the rarely used residue A (7%) at the +5 position.

Shapiro and Senapathy (1987) have described a scoring system for calculation of the likelihood that a particular splice-donor or -acceptor sequence as a whole would be functional. In their rating of 5′ splice-donor sequences, no functional donor sequences scored <60 on this scale. The mutation identified in patient TAT49 reduces the score of this splice-donor sequence from 72.8 to 49.3, suggesting that it is unlikely to be functional. The consequence of this mutation was that only *ATM* transcripts lacking exon 21 were detected by either SSCP or sequencing of cDNA from this cell line (fig. 4). Western blotting of lymphoblastoid-cell lysates from patient TAT49 failed to detect any ATM protein (data not shown).

Mutations Creating Novel Splice Sites

Three mutations were identified that created novel splice sites: IVS32-12A→G in AT11LA, 7865C→T in AT119LA, and IVS16-10T→G in AT111LA (table 2). In two of these cases, AT11LA and AT119LA, the families were consanguineous, and the mutations were homozygous, simplifying the interpretation of their effects. The IVS32-12A→G mutation created a new splice-acceptor dinucleotide site, 11 nucleotides 5′ of the normally used splice acceptor for exon 33 (diagrammed in fig. 2*C*). This mutated sequence scored high (91.6) when its potential as a splice acceptor was calculated (Shapiro and Senapathy 1987). SSCP analysis of cDNA from this region revealed several different transcripts (fig. 1*D*, bands 1–4), all of which contain an insertion of the last 11 nucleotides of intron 32. This insertion results in a frameshift predicted to cause premature truncation of the protein. The new splice-acceptor site created by the mutation was used exclusively, despite the continued presence of the normal splice acceptor downstream. The proximity of the normal and mutant splice sites suggests that preference for the mutant site may arise from a suppressing effect on the normal site. In creating a novel splice acceptor, the mutation significantly reduces the splice site–potential score for the wild-type acceptor. No ATM protein was detected in AT11LA by western blotting, consistent with the exclusive production of mutant transcripts observed by SSCP (fig. 3).

Although two of the smaller *ATM* transcripts detected in AT11LA represent the skipping of either exon 34 or exons 34 and 35 (fig. 1*D*, bands 3 and 4, respectively), control cell line LM217 (fig. 1*D*, lane N2) also produced minor transcripts with deletion of either exon 34 (fig. 1*D*, lane N2, band 3a) or exons 34 and 35 (fig. 1*D*, lane N2, band 4a), albeit without the inclusion of 11 extra nucleotides from intron 32. On the SSCP gel, an

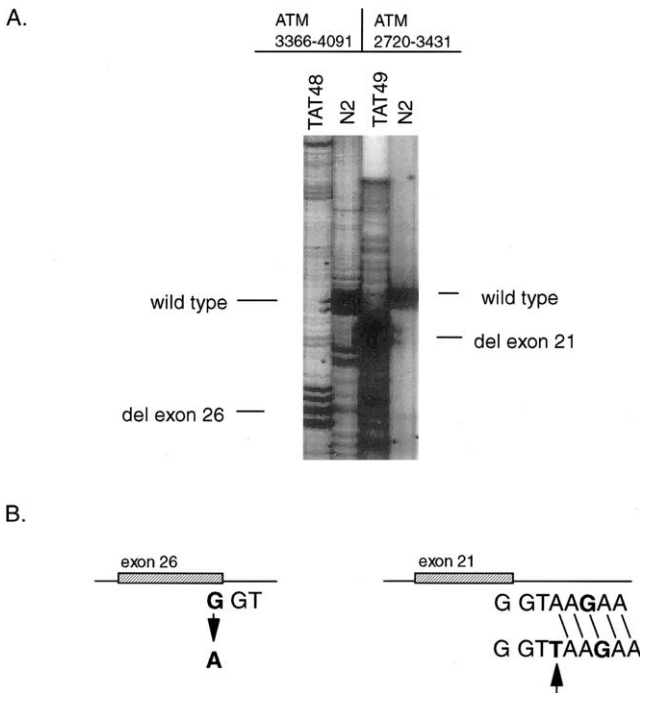

Figure 4 *A*, SSCP of *ATM* cDNA subfragments demonstrating absence of wild- type transcript in homozygous ATM patients TAT48 and TAT49. Control LM217 is represented in lane N2. Nucleotide positions of the subfragments are indicated at the top of the figure. *B*, Diagrams of the splicing consensus site defects in the genomic DNA of the patients with AT that led to the cDNA SSCP pattern in 3*A*.

additional faint band present in both the sample from AT11LA and that from control LM217 (fig. 1*D*, lane AT11LA, band 2) represents a transcript with the first 25 nucleotides of exon 34 deleted. Because no corresponding genomic mutations were detected for any of these three deleted products, and because they were found in controls, they are most likely rare, aberrantly spliced transcripts that are normally present and not the consequence of a mutation.

Patient AT119LA is homozygous for a C→T mutation at nucleotide 7865, creating a new splice-donor site within exon 55 (diagrammed in fig. 2*A*). Despite the continued presence of the normal splice-donor site, the new site was used exclusively, resulting in the deletion of 64 nucleotides at the end of the exon. Unlike the mutation in AT11LA, the novel splice site created by the mutation in AT119LA does not have an improved score for its potential as a splice site. In figure 1*A*, the SSCP pattern for this region in AT119LA displayed only the aberrantly spliced transcript. Sequencing revealed that the two major SSCP bands had 64 nucleotides deleted in exon 55. No ATM protein was detected in this cell line by western blotting (fig. 3).

Nonsense and Missense Mutations with Indirect Effects on Splicing

Two distinct nonsense mutations in exon 9 were detected in patients AT113LA (748C→T) and AT51LA (802C→T), both of whom are compound heterozygotes. Despite the fact that neither mutation altered an existing splice site or created a novel site, these cell lines produced comparable amounts of both properly spliced and exon 9–deleted transcripts (fig. 5). No additional mutations were detected in any splice-consensus sites within exon 9, including purine-rich or AC-rich splicing-enhancer sequences (reviewed by Cooper and Mattox 1997) or the ~50 bp of flanking sequences to either side. The deletion of exon 9 was also noted in two other cDNAs—that of AT140LA and that of control LM217 (fig. 5)—although in very small amounts; neither had mutations in either exon 9 or flanking introns.

Patient AT130LA is homozygous for a nonsense mutation in exon 42, resulting from the mutation 5932G→T. As shown in figure 1*E*, AT130LA produced a properly spliced transcript comparable in amount to that produced in control LM217 (fig. 1*E*, lane N2); however, AT130LA also produced a unique transcript lacking exon 42 (indicated by the arrow in fig. 1*E*, lane N2). A faint band with comparable migration to the exon 42–deleted band in AT130LA was also detected in the control cell line (indicated by the diagonal arrow in fig. 1*E*, lane N2). This band was isolated, sequenced, and found to represent a transcript lacking the first 49 nucleotides of exon 45, apparently a minor, alternatively spliced species in normal cells.

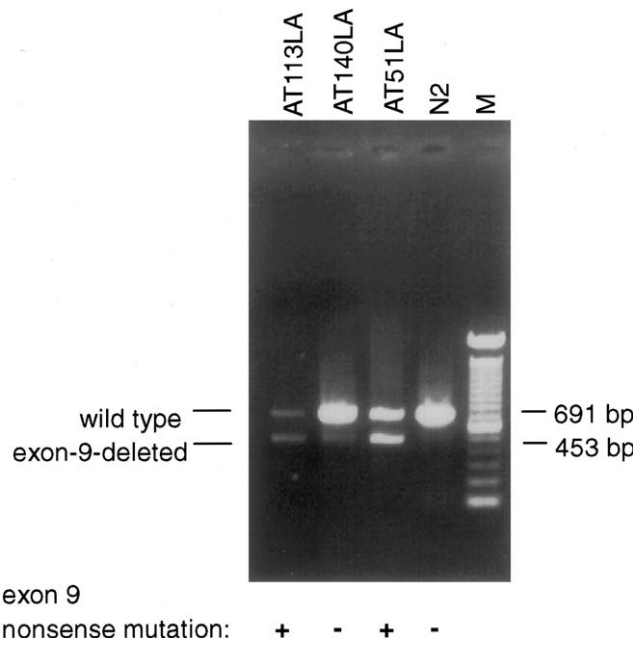

Figure 5 Missplicing of exon 9, in the absence of splice-site mutations. Shown are nested-PCR products of *ATM* nucleotide positions 500–1191 from cDNAs of AT113LA, AT140LA, and AT51LA and from control LM217 (lane N2). Patients AT113LA and AT51LA are heterozygous for the exon 9 nonsense mutations 748C→T and 802C→T, respectively. AT140LA and control LM217 have no mutation in either exon 9 or the flanking splice consensus sites. All cell lines produce varying amounts of transcripts with exon 9 deleted. The 123-bp ladder marker is shown in lane M.

AT138LA produced only *ATM* transcripts with exon 44 deleted. No other evidence of a mutation elsewhere in the gene was detected by PTT. Sequencing of exons 43–46 and flanking intronic sequences revealed only a single mutation, 6154G→A, within exon 44, resulting in a nonconservative amino acid substitution, 2032E→K. Deletion of exon 44 results in a frameshift. Western blotting of lysates from this cell line failed to reveal any ATM protein (data not shown).

Variant Splicing Not Associated with Mutations

During the course of screening *ATM* cDNA subfragments for mutations, several regions were encountered where transcripts had exon(s) or portions of exons deleted but for which no underlying genomic mutations within either the exon or the flanking splice sites could be found; for example, PTT assays revealed aberrantly migrating bands in the region encompassing portions of exons 10–12 in patients AT115LA, AT81LA, and AT139LA. The cDNA from these patients contained substantial amounts of products lacking exon 11, as shown in the SSCP assay in figure 6 and as confirmed by isolation and sequencing of the shortened products. However, no splice-site mutations were detected flanking

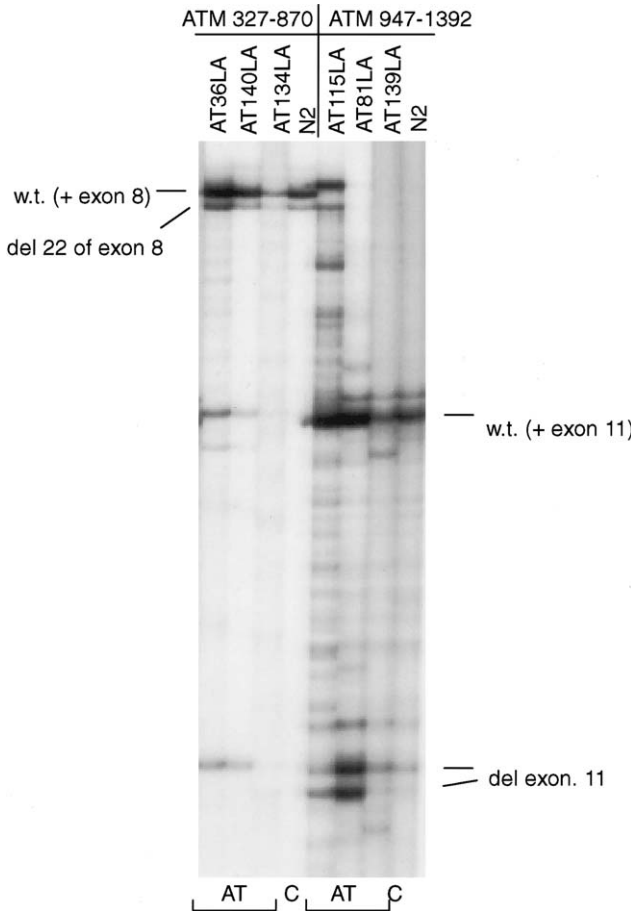

Figure 6 Variant splicing of *ATM* transcripts, in the absence of mutations. Nested PCR of *ATM* nucleotides 327–870 from three patients with AT and from control LM217 (lane N2) show an alternatively spliced transcript missing the first 22 nucleotides of exon 8. Nested PCR of *ATM* nucleotides 947–1392 from three patients and the control LM217 (N2) show an alternatively spliced transcript with exon 11 deleted. Patient AT139LA had bands below the wild-type and exon 11–deleted bands, representing its mutation in exon 10, as 1024delAAAG. Patients AT115LA and AT81LA are homozygous and heterozygous, respectively, for an exon 12 mutation (1563delAG) not contained in the amplified fragment.

exon 11 in the genomic DNA of these individuals. Examination of surrounding exons did reveal truncating mutations in three of these patients: AT115LA and AT81LA had a deletion of two nucleotides in exon 12, and AT139LA had a deletion of four nucleotides in exon 10 (table 2). Transcripts lacking exon 11 were also readily detected in control LM217 (fig. 6), again without any underlying mutation being detected.

Amplified cDNAs from cell lines of patients with AT that had truncated fragments in the PTT assay for the region containing exons 7–10 also displayed a wide variety of bands on SSCP gels (fig. 6). The cDNA from patients AT45LA, AT51LA, AT84LA, AT113LA, AT134LA, and AT140LA contained a product lacking the first 22 nucleotides of exon 8. The presence of an AG dinucleotide at the end of the 22 deleted nucleotides is consistent with the use of a cryptic splice-acceptor sequence. However, there was no mutation of the original splice acceptor in the genomic DNA of any of the individuals. Moreover, cDNA from control LM217 also contained the 22 nucleotide–deleted product (fig. 6).

Other examples of cryptic-splice-site utilization were seen in minor transcripts from a number of patients with AT and from controls. These include the deletion of the first 41 nucleotides of exon 15 (in AT117LA), the deletion of the first 86 nucleotides of exon 14 (in AT63LA), and either the skipping of exons 11, 34, and/or 35 or deletion of the first 19 nucleotides of exon 17 (in control LM217). In all these cases, no splice-site mutations were detected in the genomic DNA; however, the sequences at the end of the cDNA deletions indicated that cryptic splice sites had been used.

Discussion

Most studies of *ATM* mutations suggest that a high percentage are inactivating mutations resulting in protein truncation and the production of little or no ATM protein. In the *ATM* gene, with its large complement of exons, splice sites represent a sizable target for mutations that would have severe effects on the protein product. In a previous review of AT mutations (Concannon and Gatti 1997), we noted that a high percentage (39%) of alterations detected in *ATM* cDNA from patients with AT reflected the loss of one or more exons, a result consistent with this hypothesis. However, because the majority of published studies have examined only cDNA transcripts of *ATM*, there is a potential for ascertainment bias in the reported number of splicing mutations in the *ATM* gene. The loss of an entire exon is easily apparent by visual examination of enzymatically amplified cDNA products, regardless of what mutation-screening technique is being used. Therefore, exon skipping should be among the defects easiest to identify in cDNA. Furthermore, in cases in which an underlying genomic mutation has not been identified, exon skipping may simply reflect low-level missplicing that occurs normally but is more apparent in AT cell lines in which overall *ATM* transcript levels may be reduced.

In the set of 62 *ATM* mutations reported in the present study, a significant fraction (30/62 [48%]) affected splicing of the ATM transcript. These results are consistent with our earlier suggestion that splicing-related mutations are unusually frequent in AT. The canonical GT and AG dinucleotides that flank most mammalian exons are frequent targets of mutations that affect splicing (59% of mutations compiled by Krawczak et al. [1992] and 71% of mutations compiled by Schwarze et al. [1997]). In contrast, the majority of mutations effecting splicing that were detected in the present study did not

directly alter the canonical AG or GT exon-flanking dinucleotides but, instead, either targeted less-conserved sequences surrounding splice junctions or created novel splice sites. Several other instances of exon skipping that were observed in ATM were not associated with any detectable underlying mutation, suggesting that some degree of caution is necessary in the interpretation of such alterations when they are observed only in cDNA.

Two different mutations detected in the present study, 3576G→A and IVS40+1126A→G, have previously been described (by Gilad et al. [1998] and McConville et al. [1996], respectively) in patients reported to exhibit an atypical clinical course of AT. The mutation 3576G→A in patient TAT48 resulted in exclusive production of transcripts with exon 26 deleted. Deletion of exon 26 does not alter the translational reading frame for the ATM protein. No normal-length transcripts were detected, despite the sensitivity of PCR as an assay. If an exon 26–deleted transcript were to be translated into a stable protein, it would be only 58 amino acids (~2% of the total length) shorter than normal. On an SDS-PAGE gel, which is commonly used for protein assays, such a protein would be indistinguishable from the normal one. Gilad et al. (1998) have described several cell lines that are homozygous for this mutation and in which they could detect a low level of ATM protein (~5% of the level in controls). In the present study, no ATM protein was detected in patient TAT48. The discrepancy between our results and those of Gilad et al. may be due to differences in experimental conditions. However, in light of the absence, in patient TAT48, of any transcripts containing exon 26, only ATM protein with an internal deletion of 58 amino acids (encoded by exon 26) could be produced. If such a shortened protein is produced in these cells, it is unclear whether it would be functional or have any significant impact on the clinical phenotype.

An additional patient in the present study, AT117LA, was heterozygous for the 3576G→A mutation and had clinical features consistent with the AT variant disorder AT_{Fresno}. AT_{Fresno} encompasses all of the features of AT but includes microcephaly and mental retardation in the phenotype (Curry et al. 1989). AT117LA is only the second patient with AT_{Fresno} who has been screened for *ATM* mutations. The other previously characterized AT_{Fresno} patient, AT25LA, is homozygous for a splice-site mutation in intron 33 (Gilad et al. 1998). The 3576G→A mutation in AT117LA has been observed in several patients with AT who have classic phenotypes, including two patients (AT142LA and TAT48) in the present study. The IVS33+2T→C mutation in AT25LA is unique, but there are several patients with AT who have a very similar IVS33+2T→A mutation, one of whom is homozygous. Neither AT117LA nor AT25LA produces detectable levels of ATM protein. Thus, the variant phenotype observed in these patients with AT_{Fresno} seems unlikely to result from the nature of their *ATM* mutations alone, given (1) the occurrence of these same mutations in typical patients with AT and (2) the fact that absence of detectable ATM protein is also frequent among classic patients with AT. The AT_{Fresno} phenotype may result instead from epistatic effects of other, as yet unidentified genes.

The IVS40+1126A→G mutation, reported to be associated with a milder clinical course of AT (McConville et al. 1996), was detected in two patients (AT56LA and CAT13; fig. 1*F*) in the present study and in one patient (AT13LA) from a previously published study (Wright et al. 1996), all of whom are compound heterozygotes. In AT56LA, the two *ATM* alleles could be distinguished because of a nearby common polymorphism (5557G/A in exon 39; Dork et al. 1997). The allele containing the IVS40+1126A→G mutation produced both wild-type and mutated (insertion of 137 nucleotides) transcripts in comparable, albeit low, amounts. However, no *ATM* protein was detected in any of these cell lines (see fig. 3, lanes AT13LA and AT56LA), raising concerns as to whether the production of this small amount of normal transcript could significantly affect the phenotype of these patients.

All 12 patients whom McConville et al. (1996) described as having a milder clinical phenotype were compound heterozygotes for the IVS40+1126A→G mutation, as were patients AT56LA, CAT13, and AT13LA. However, in 8 (67%) of these 12 patients, the ages at onset of ataxia were 1–3 years, which is quite characteristic of classic AT. The age at onset of ataxia in the three affected siblings in the family of patient AT56LA were also characteristic of AT: 8 mo, 18 mo, and 3 years. In the family of patient AT13LA, the ages at onset were 5 years and 6 years for the two affected siblings. McConville et al. (1996) also suggest a decreased frequency of cancer in IVS40+1126A→G patients. This is corroborated in the families of patients AT56LA and AT13LA; in the latter, the affected siblings lived to ages 40 and 44 years without cancer. Thus, some aspects of the milder phenotype reported in conjunction with the IVS40+1126A→G mutation appear to recur in separate patient groups. However, identification and clinical characterization of additional patients with this mutation will be necessary to clarify the exact relationship between phenotype and genotype.

Twelve (~20%) of the patients in the present study had mutations that resulted in nonsense premature-termination codons (PTCs), and in five of these patients the PTCs were associated with some degree of deletion of the exon containing them. All prokaryotic and eukaryotic cells that have been examined appear to have mechanisms to degrade RNAs that harbor PTCs (Maquat 1995, 1996). However, in cell lines from patients with AT who are compound heterozygotes for a PTC

mutation and a second unrelated mutation, equimolar amounts of mRNA from PTC-containing alleles and from the other allele are detected by reverse transcriptase–PCR. The observed skipping of the *ATM* exon containing the PTC appears to be a specific event, one that is not associated with general degradation of transcripts containing the PTC. PTC mutations that lead to specific deletion of the exons containing them have also been reported for *BRCA1* (Mazoyer et al. 1998), the gene for fibrillin (Dietz et al. 1993), *CFTR* (Hull et al. 1994), the gene for mouse immunoglobulin κ (Aoufouchi et al. 1996), and the gene for mouse urinary protein (Belgrader and Maquat 1994), among others. Reports that even missense mutations can cause the deletion of exons containing them (Belgrader and Maquat 1994; Liu et al. 1997) suggest that additional mechanisms, not dependent on the presence of PTCs, may lead to effects on splicing.

In the present study, one patient (AT138LA) had only *ATM* transcripts with exon 44 deleted. Examination of the genomic sequence in and around exon 44 revealed only a single homozygous change, a G→A substitution at position 6154, a position that is conserved in the mouse *ATM* sequence. The resulting nonconservative amino acid substitution, E→K, may alter protein structure or function, but it would not necessarily be expected to terminate protein translation. Despite the observed missplicing of exon 44, this mutation does not affect any known splicing-control motif (including exonic splicing-enhancer sequences; see review by Cooper and Mattox [1997]). Hoffmeyer et al. (1998) have suggested that particular PTCs, while not altering any established splice-consensus sites, may cause defective splicing by inducing a significant alteration in the secondary structure of the transcript, disrupting the formation of the spliceosome. In a study of mutations in the *HPRT* gene, Steingrimmsdottir et al. (1992) noted that all of seven missense mutations that were associated with splicing defects disrupted base pairing in stem-loop structures in the predicted secondary structure of the corresponding RNA. In light of the observations, in AT138LA, of both a deletion of exon 44 and the 6154G→A missense mutation, it seems reasonable to postulate that this missense mutation may also cause defective splicing, by a similar mechanism. When the RNA secondary structure of exon 44 was predicted by the program mfold, position 6154 was projected to lie at the base of a stem-loop structure. The G→A mutation in AT138LA disrupts the GC base pair at this site, resulting in a predicted destabilization of a portion of the stem structure, a result consistent with the model of Steingrimmsdottir et al. (1992)

For some regions of *ATM,* exon-deleted transcripts have been detected even in controls. Although multiple, differentially spliced transcripts have been described for *ATM,* all were due to alternative splicing in the untranslated regions, not in the coding exons (Savitsky et al. 1997). Because the *ATM* mRNA is large, and because mutation screening involves amplification of only portions of the mRNA at a time, we cannot rule out the possibility that alternatively spliced products described in the present study are derived from either nonfunctional, truncated transcripts or transcripts in the process of being degraded. However, regardless of whether they play a functional role, the fact that these exon-deleted products are easily detected in significant amounts in standard mutation-screening assays indicates that caution should be applied in the interpretation of observed cases of exon skipping in *ATM* cDNA, until a credible underlying mutation in genomic DNA can be identified.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Ataxia-Telangiectasia Mutations Database, http://www.vmmc.org/vmrc/atm.htm
mfold, http://www.ibc.wustl.edu/~zuker/rna/form1.cgi
Online Mendelian inheritance in man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for AT [MIM 208900])
Recommendations for a Nomenclature System for Human Gene Mutations, http://ariel.ucs.unimelb.edu.au:80/~cotton/antonara.htm

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