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Insertion in the mRNA of a metachromatic leukodystrophy patient with sphingolipid activator protein-1 deficiency

(lysosomal disease/sulfatide/polymerase chain reaction/sulfated glycoprotein 1/alternative splicing)

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ABSTRACT **The lysosomal catabolism of sulfatide requires arylsulfatase A and a specific sphingolipid activator protein, SAP-1. While most patients with metachromatic leukodystrophy have mutations in the gene for arylsulfatase A, some patients have deficient SAP-1, as determined by immunological techniques. We now describe the molecular findings in a patient who died at 22 years of age with SAP-1 deficiency. The DNA polymerase chain reaction was used to amplify regions of cDNA which were subcloned in M13 phage DNA and sequenced by the dideoxy chain-termination method. The patient was found to have a 33-base-pair insertion between nucleotides 777 and 778 (numbered from the A of the ATG initiation codon). No other changes were found in the coding sequence of the cDNA from this patient. At the site of the insertion some normal people have an additional 9 base pairs, which correspond to the last 9 nucleotides at the 3′ end of the insertion. The cDNAs from the second-cousin parents were amplified and sequenced, and in both two alleles were identified, one with the 33-base-pair insertion and one with no insertion. Two brothers were found to have only the normal alleles and a sister was found to have the 33-base-pair insertion and a normal allele. The findings confirm studies performed on leukocyte extracts demonstrating normal antigen levels in the two brothers and a lower level in the sister. The presence of 11 additional amino acids in the coding region of mature SAP-1 in this patient causes significant changes in the hydropathy profile compatible with the previous findings at the protein level.**

The catabolism of most sphingolipids requires both a specific lysosomal enzyme and a specific, relatively low molecular weight protein we call sphingolipid activator protein (SAP) (1). While at least six SAPs have been identified, the exact number is not known. However, it is now obvious that, while most lysosomal disorders are due to defects in lysosomal enzymes, some very serious and fatal disorders are due to defects in SAP (2–5). These include the AB variant of GM2 gangliosidosis, a variant form of metachromatic leukodystrophy, and a variant form of Gaucher disease. While the patients have clear evidence for decreased catabolism of a specific lipid, the lysosomal enzyme activity measured *in vitro* is normal and there is evidence for decreased levels of a specific SAP in all cases thus far identified (2, 5, 6).

The cDNA coding for the SAP-1 precursor species, called prosaposin by Morimoto *et al.* (7), has been cloned and sequenced (8, 9). Most recently Nakano *et al.* (10) published the complete corrected sequence, which is in agreement with our unpublished data, except those authors found a 9-base-pair (bp) insertion between nucleotides 777 and 778 (counting from the A of the ATG initiation codon) in clones from normal people. The SAP-1 precursor protein contains four domains of about 80 amino acids, each with similar structural features, including glycosylation sites and proline and cysteine residues (11–13). The domains are proteolytically cleaved during biosynthesis to yield the four potential SAPs that can stimulate different hydrolytic reactions. Both SAP-1 (domain 2) and SAP-2 (domain 3) are coded for by this gene, as well as saposin A (domain 1) (14) and component C (domain 4) (11). Previous studies in this laboratory had determined that both SAP-1 and SAP-2 were produced from 70-kDa precursors (15, 16), both mapped to human chromosome 10 (17, 18), and patients with SAP-1 deficiency had detectable SAP-2 (14). We now know that one gene codes for SAP-1 and SAP-2, so the mutation causing SAP-1 deficiency in some patients must not affect the next downstream domain.

We now describe a mutation in mRNA found in the patient described by Hahn *et al.* (4). A 33-nucleotide insertion, including the 9 base pairs reported by Nakano *et al.* (10), was found in cDNA amplified by the polymerase chain reaction (PCR) and sequenced in M13 phage DNA. This change, the only one found on sequencing the entire cDNA of the patient, caused a significant change in the structure of mature SAP-1 that could explain the findings at the protein and RNA levels reported previously (15, 16, 19–21).

MATERIALS AND METHODS

Materials. Restriction endonucleases and M13 mp18 and -19 replicative form DNA were purchased from Boehringer Mannheim. Modified T7 DNA polymerase (Sequenase) and M13 sequencing primers were from United States Biochemical. Thermostable *Thermus aquaticus* (*Taq*) DNA polymerase was obtained from Perkin–Elmer/Cetus. Deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate was purchased from New England Nuclear. Oligonucleotide primers were synthesized in the Jefferson Department of Biochemistry and Molecular Biology on a DuPont Coder 300 DNA synthesizer.

Cell Lines. Initial studies were performed on cultured skin fibroblasts from the patient of Hahn *et al.* (4) generously supplied by Bruce A. Gordon (London, Ontario). Studies on the family members were done on transformed lymphoblasts prepared in this laboratory from 8 ml of heparinized blood. Cultured skin fibroblasts were grown in Eagle's minimal essential medium supplemented with 15% fetal bovine serum, 2 mM glutamine, and 1% penicillin/streptomycin (GIBCO). Lymphoblasts were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and 1% gentamycin. Previous studies on the cultured skin fibroblasts from this patient revealed decreased ability to metabolize ^{14}C-labeled sulfatide (4), decreased amount of mature SAP-1 (16, 19), and near-normal level of mRNA (21).

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Abbreviations: SAP, sphingolipid activator protein; PCR, polymerase chain reaction.

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Isolation of RNA. Total RNA was isolated from the cultured fibroblasts and lymphoblasts by using the guanidinium isothiocyanate/cesium chloride procedure (ref. 22, p. 196). Messenger RNA was eluted from an oligo(dT) column (New England Biolabs), using instructions supplied by the manufacturer.

Synthesis of cDNA. Double-stranded cDNA was synthesized from total RNA or mRNA by using the kit purchased from Bethesda Research Laboratories according to the manufacturer's instructions. The cDNA produced was precipitated with ethanol and dissolved in water.

Isolation of Genomic DNA. Genomic DNA was isolated from the cultured skin fibroblasts and lymphoblasts according to a method modified from Maniatis *et al.* (ref. 22, pp. 280–281).

Amplification of cDNA and Sequencing of PCR Products. Using a series of oligonucleotide primers, starting from the ATG initiation codon and continuing to the last nucleotide (no. 2717) before the poly(A) tail, we amplified the full length of the cDNA from the patient. The primers were synthesized with addition of a endonuclease restriction site (either *Pst* I or *Eco*RI) if a naturally occurring site was not present. Additional oligonucleotide primers were synthesized and used to amplify regions of the cDNA so that overlap in all segments, including natural restriction sites, was obtained. The locations of the initial oligonucleotide primers used are illustrated in Fig. 1. Their exact structure is available upon request. PCR amplification was carried out on a Perkin–Elmer/Cetus Thermal cycler using the following conditions: initial denaturation at 96°C for 10 min, addition of 2.5 units of *Taq* polymerase, denaturation at 94°C for 1 min, annealing at 52°C for 1 min and extension at 72°C for 1 min, for 25 cycles, followed by storage at 4°C until processing. The amplified DNA was extracted with phenol/chloroform, precipitated with ethanol, and dissolved in water. After the amplified regions had been cut with *Pst* I and *Eco*RI and purified on 2.5% low-melting-temperature agarose, they were subcloned in M13 vectors (mp18 and mp19) and sequenced by using the Sanger dideoxy chain-termination method (23) with appropriate sequencing primers and deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate. Products of the sequencing reaction were electrophoresed at 60 W on 6% polyacrylamide gels. After drying, the gel was placed in contact with Kodak X-Omat AR film at −70°C overnight.

PCR Amplification of cDNA and Genomic DNA. Two PCR primers spanning 111 nucleotides in "normal" cDNA encompassing the region of the insert were synthesized and used to amplify cDNA and genomic DNA from the patient and her family. These primers have the following sequences: (111+) 5′-ATTGCTATCCAGATGATGAT-3′ and (111−) 5′-CACTTTGGCGGGGACCAGAG-3′. The PCR reaction was carried out as described above. After amplification, an aliquot was electrophoresed in 4% agarose and stained with ethidium bromide along with low molecular weight DNA standard (*Hae* III digest of φX174 phage DNA) (New England Biolabs).

Measurement of Antigen Levels in Leukocytes from the Patient and Family Members. Leukocytes were prepared from 8 ml of heparinized blood, and SAP-1 was measured in the supernates from the sonicates of the leukocyte pellets by using rocket immunoelectrophoresis as described previously (24).

Hydropathy Profile. The deduced structures of mature SAP-1 with and without insertions were analyzed by using the method of Kyte and Doolittle (25) on an IntelliGenetics program.

RESULTS

Previous studies using cultured skin fibroblasts from this patient revealed decreased ability to metabolize ^{14}C-labeled sulfatide *in situ* (4), normal arylsulfatase A activity (4), no detectable immunoreactive bands in the region of mature SAP-1 on Western blotting (19), production of a precursor species that could be isolated from the medium when the cells were labeled with [^{35}S]methionine for 24 hr in the presence of 10 mM NH_4Cl (21), and near-normal levels of mRNA (21). On the basis of the above findings, the cells from the now-deceased patient were examined for changes within the coding region of the mRNA.

Initial attempts to prepare a cDNA library from the cells of this patient were unsuccessful due to their poor growth characteristics—they failed to produce sufficient cells to obtain a representative library. Using a series of PCR primers to amplify the full length of the cDNA, we could determine the sequence. The initial region selected for amplification and sequencing was the region encompassing primers C to D′ (Fig. 1), which included the total coding domain of mature SAP-1. These primers have the following sequences: C, 5′-TCGACTGCAGGAGCTGGACATGACTGAGGT-3′; and D′, 5′-CACCAGGAATTCACACACCT-3′ (the underlined portion of the C primer includes the added *Pst* I restriction site). As shown on Fig. 2 there was a 33-nucleotide insertion between nucleotides 777 and 778 in the normal cDNA sequence (counting from the A of the ATG initiation codon). PCR of this region was performed four times with different batches of cDNA prepared from total RNA and mRNA from this patient. The DNAs of more than 50 clones

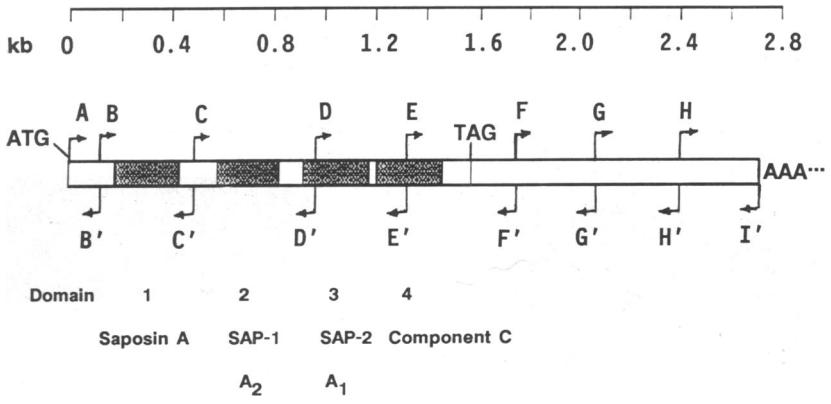

FIG. 1. Key features in the SAP-1 precursor cDNA and locations of the primary PCR primers. The cDNA was sequenced from the ATG initiation codon to the last nucleotide (2717) before the poly(A) tail. The shaded regions highlight the four SAP domains (with commonly used nomenclature given below each one). The letters above the bar denote primers in the positive orientation and those below the bar, in the negative orientation. kb, Kilobases.

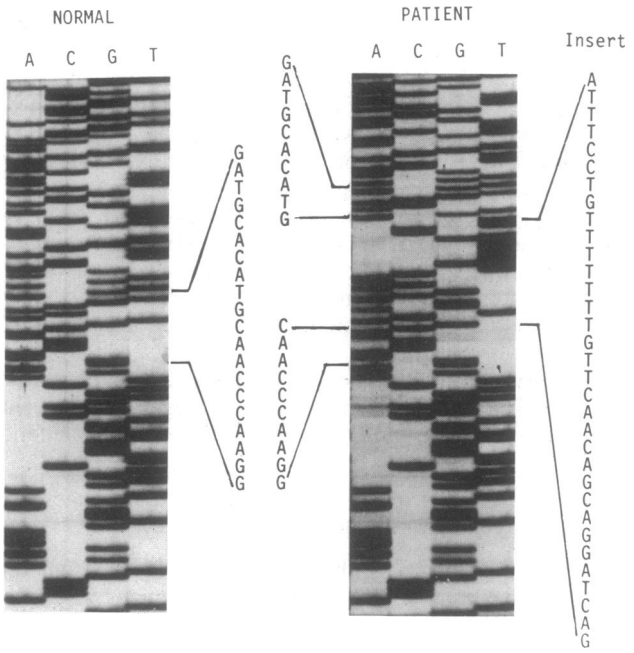

FIG. 2. Sequence analysis of the patient and a control, demonstrating the insertion in the cDNA from the patient. The normal sequence given on the left side starts from nucleotide 787 on the bottom to 767. The sequence of the insertion is given on the right.

were sequenced in both orientations. In 48/54 clones the 33-nucleotide insert was obtained, and in 6/54 clones a 57-nucleotide deletion was obtained that eliminated the normal nucleotides from 721 to 777 but left the 9 nucleotides found at the 3′ end of the 33-nucleotide insertion. No other changes were found in this region or in any other region of the total cDNA structure (data not shown).

When new primers were used to amplify a 111-bp fragment encompassing the region of interest, the insertion in the patient could be clearly visualized on ethidium bromide staining of PCR products (Fig. 3). PCR amplification of this area using cDNA from the parents and normal siblings revealed a pattern completely consistent with the autosomal recessive pattern of inheritance of this disorder and the consanguinity within this family. As shown in Fig. 3 the parents, who are second cousins, have two products, one with an insertion of 33 nucleotides and one without. The two brothers show only the normal allele and the sister shows the two alleles like the parents. Studies performed previously but never published using our antibodies to measure the amount

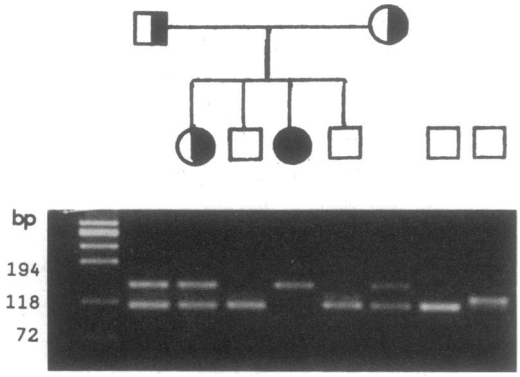

FIG. 3. Partial pedigree of this family and PCR analysis of the tested members and two controls. PCR primers spanning 111 nucleotides in normal cDNA in the regions of the insertion were used to amplify cDNA from all samples. The left lane is the DNA molecular weight marker.

Table 1. Level of SAP-1 in leukocyte extracts as measured by rocket immunoelectrophoresis

Subject	SAP-1, μg/mg solubilized protein	Genotype assignment
Patient	Undetectable	Affected
Mother	0.79	Obligate carrier
Father	0.87	Obligate carrier
Sister	1.13	Carrier
Brother 1	1.36	Noncarrier
Brother 2	1.77	Noncarrier
Controls (n = 41)	1.35 ± 0.27	

of SAP-1 antigen in leukocyte extracts clearly identified the parents as carriers, the two brothers as noncarriers, and the sister as a probable carrier (Table 1). The data in Fig. 3 are consistent with the genotype assignment made previously. The regions of the insertion from the parents and the sister were sequenced, and they contain the same 33 nucleotides found in the patient (data not shown). One of the two controls shown has the extra 9 nucleotides reported to occur in normal individuals (10).

In an attempt to develop a rapid test for this mutation in this family, genomic DNA from lymphoblasts and cultured skin fibroblasts was isolated and amplified by using the primers that span 111 nucleotides encompassing the site of insertion. Amplification was successful, in that a region identical in size to the normal cDNA product was obtained (Fig. 4). However, no insertion could be found in the genomic DNA of the patient or in her parents or siblings. Sequencing of an amplified fragment from the genomic DNA of the patient revealed only normal cDNA sequence. This is probably explained by the existence of an intronless pseudogene present for this region that resulted in the preferential amplification of the intronless sequence and no amplification of the intron-containing true gene when those primers were used.

The finding of the 33-nucleotide insertion in the SAP-1 coding domain of the patient is consistent with our previous findings at the protein and mRNA levels. While some normal people have an extra 9 base pairs between nucleotides 777 and 778 (see Fig. 3 and ref. 10), the effect of the three additional amino acids on the protein structure in the SAP-1 domain is minimal (Fig. 5). Insertion of 24 nucleotides in addition to these 9 nucleotides (as is found in this patient), coding for a total of 11 additional amino acids, produces significant changes in the hydropathy profile (Fig. 5) which could alter the stability of the SAP-1 domain, leaving other domains apparently unaffected.

DISCUSSION

PCR-amplified cDNA from a patient previously diagnosed with a variant form of metachromatic leukodystrophy caused

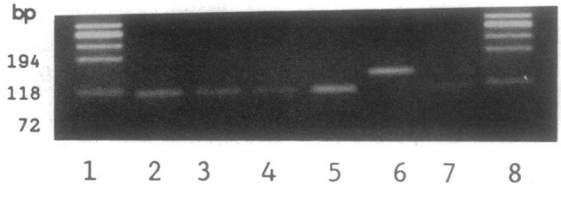

FIG. 4. PCR analysis of genomic DNA and cDNA from this family and controls. Two primers spanning 111 nucleotides in normal cDNA encompassing the insertion were used to amplify genomic DNA from two controls (lanes 2 and 7), the patient (lane 5), her father (lane 3), and her mother (lane 4) and cDNA from the patient (lane 6). Lanes 1 and 8 are DNA molecular weight markers.

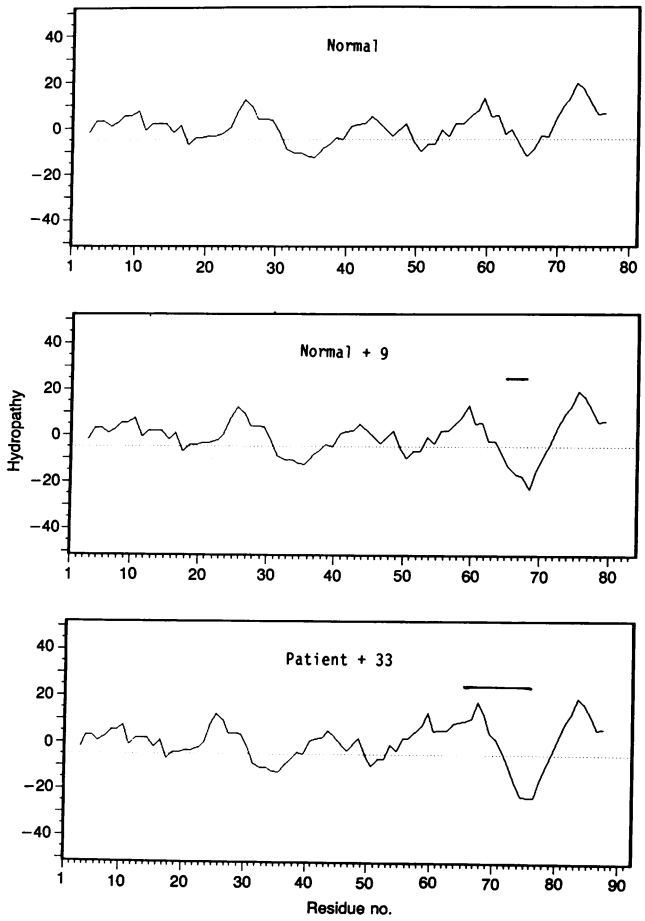

FIG. 5. Hydropathy profile of the mature SAP-1 domains in normal, normal plus 9 nucleotides, and patient with 33 additional nucleotides. The curves were drawn by computer based on the algorithm described by Kyte and Doolittle (25). The horizontal bars in the lower two panels span the region of the amino acid insertions.

by a deficiency of mature SAP-1 was found to have a 33-bp insertion in the SAP-1 coding domain. This 33-bp insertion located between nucleotides 777 and 778 includes 9 base pairs reported to occur in normal cDNA by Nakano *et al.* (10) and confirmed by PCR amplification of control cDNA in this study (Fig. 3). The region surrounding the insertion site is 100% nucleotide identical between normal human SAP-1 cDNA and the sulfated glycoprotein 1 cDNA found in rat Serotoli cells reported by Collard *et al.* (26), indicating strong species conservation in this region of the coding sequence of these related genes (Fig. 6). The additional 24 nucleotides code for 8 amino acids, including many hydrophobic species. These in turn cause a significant change in the hydropathy profile (Fig. 5) that probably leads to decreased stability of the partially processed SAP-1, as has been shown for more subtle amino acid changes (27, 28). In addition, the two-dimensional model of mature SAP-1 proposed by O'Brien *et al.* (12) would be significantly altered by the addition of 8 or 11 amino acids near the site of the helix-breaker proline at amino acid position 68 in the mature SAP-1. The last proline and two remaining cysteine residues are completely conserved in the four SAP domains found in the SAP precursor (12).

This insertion in the patient's cDNA was the only change found on sequencing the entire structure. The cells from this patient had previously been shown to have very low levels of cross-reacting antigen (19) and near-normal levels of mRNA (21). Evidence for the presence of mature SAP-1 after [^{35}S]methionine labeling of cells and immunoprecipitation with monospecific antiserum was not found after SDS/PAGE and exposure to x-ray film (16). However, when cells were given 10 mM NH_4Cl along with the [^{35}S]methionine, the precursor species could be found in the medium. On reevaluation of the gels it does appear that the glycosylated ≈70-kDa precursor species is slightly larger than normal (data not shown). Treatment of the radiolabeled precursor with trypsin and chymotrypsin resulted in a more unstable species than normal and some unique polypeptide fragments on SDS/PAGE. These data would suggest that some proteolytic processing of precursor SAP-1 may take place before it reaches the lysosome and the additional amino acids may make the mature SAP-1 unstable and rapidly degraded. In addition, it is possible that the additional 11 amino acids change the configuration such that our antibodies are less reactive toward the antigenic sites on SAP-1.

Further confirmation of the significance of the insertion is the finding of this allele, plus the normal allele, in the cDNA from the related parents. The fact that two brothers called noncarriers by antigen analysis had only the normal allele and the sister called a probable carrier by that test had the mutant and normal allele provides further evidence that this mutation causes SAP-1 deficiency in this family (Fig. 3). cDNAs from more than 10 controls have been examined, and although some have the 9-bp insertion in this region none have any additional nucleotides. Three other patients from two families with SAP-1 deficiency (3, 29) do not have this mutation (unpublished observation).

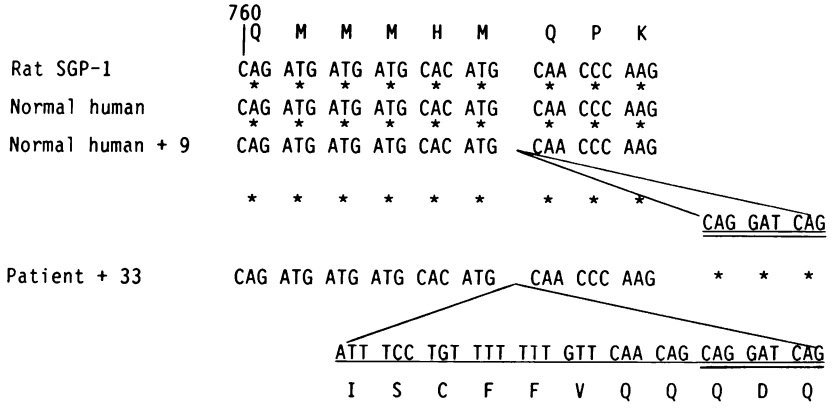

FIG. 6. Nucleotide and amino acid sequences (standard one-letter code) showing complete homology on either side of the insertion site between rat sulfated glycoprotein 1 (SGP-1), normal human, normal human plus 9, and patient plus 33. Rat SGP-1 sequence is from Collard *et al.* (26) and normal human plus 9 sequence is from Nakano *et al.* (10). Asterisks between lines denote identical codons; the singly underlined sequence is unique to this patient and the doubly underlined sequence is found in some controls and the patient.

These studies raise some additional questions. Where does the 33-bp insertion come from? Is the 9-bp insertion a polymorphism or some form of alternative RNA splicing taking place under certain growth conditions? Why do a few clones from the patient have 57 nucleotides deleted, leaving the last 9 nucleotides of the insertion? Protein sequencing has not revealed the three amino acids coded for by the 9 base pairs in normal human SAP-1 or in rat sulfated glycoprotein 1 (26, 30). A search for the 33 base pairs in GenBank on April 1, 1989, did not yield any clue as to its source. It is proposed that this insert comes from an intronic sequence within the gene for SAP-1 precursor. A single base mutation within the intronic sequence may cause altered splicing to add the 33 base pairs to the normal mRNA. Examination of the sequence of the 33-nucleotide insertion (Fig. 6) reveals several features consistent with this being the 3′ end of an intron. Several AG pairs are found including one at the end, one four nucleotides away, and one seven nucleotides away. This region is preceded by a stretch of thymidines. Alternative splicing to these three AG pairs could result in either no extra nucleotides, nine extra nucleotides [as reported by Nakano *et al.* (10)], or six extra nucleotides [recently found in normal cDNA (unpublished data)]. The few clones from the patient that have the 57-nucleotide deletion may have undergone a secondary effect of the mutation that alters normal splicing, as has been demonstrated for other splice junction defects (31). However, proof for these theories will come only with sequencing of the introns in the region of the insertion. Further studies on the gene and pseudogene will yield interesting findings on this disease and the role this gene plays in the synthesis of multiple SAP and sulfated glycoprotein.

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