HHS Public Access

Author manuscript

J Immunol. Author manuscript; available in PMC 2021 September 15.

Published in final edited form as:

J Immunol. 2020 September 15; 205(6): 1535–1539. doi:10.4049/jimmunol.2000272.

A POINT MUTATION CREATING A 3′ SPLICE SITE IN C8A IS A PREDOMINANT CAUSE OF C8α-γ DEFICIENCY IN AFRICAN AMERICANS

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Abstract

C8α-γ deficiency was examined in four unrelated African Americans. Two individuals were compound heterozygotes for a previously reported point mutation in exon 9. mRNA from the remaining six C8A alleles contained a 10 nt insertion between nt 992 and 993 corresponding to the junction between exons 6 and 7. This suggested that C8α-γ deficiency in these individuals was caused by a splicing defect. Genomic sequencing revealed a G→A point mutation in intron 6, upstream of the exon 7 acceptor site. This mutation converts a GG to an AG, generates a consensus 39 splice site that shifts the reading frame, and creates a premature stop codon downstream. To verify that the point mutation caused a splicing defect, we tested wild-type and mutant mRNA substrates, containing 333 nt of the C8α intron 6/exon 7 boundary, in an *in vitro* splicing assay. This assay generated spliced RNA containing the 10 bp insertion observed in the C8α mRNA of affected patients. In addition, in mutant RNA substrates, the new 39 splice site was preferentially recognized compared with wild-type. Preferential selection of the mutant splice site likely reflects its positioning adjacent to a polypyrimidine tract that is stronger than that adjacent to the wild-type site. In summary, we have identified a G→A mutation in intron 6 of C8A as a predominant cause of C8α-γ deficiency in African Americans. This mutation creates a new and preferred 39 splice site, results in a 10 nt insertion in mRNA, shifts the reading frame, and produces a premature stop codon downstream.

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INTRODUCTION

Complement dependent bactericidal activity, a vital component of innate immunity, develops upon assembly of the membrane attack complex from the terminal complement (C) components, C5-C9. A deficiency of any of the C5-C9 terminal complement components is associated with loss of serum bactericidal activity and increased risk of meningococcal disease (1, 2). One of the terminal components, C8, is composed of three polypeptide chains (α, β and γ), each encoded by a separate gene: A, B and G, respectively (3, 4). Mature, functionally active C8 contains two non-covalently associated subunits: C8β and C8α-γ. In contrast, the two polypeptide chains of C8α-γ subunit are covalently linked by a disulfide bond (5).

Two C8-deficiency states have been described corresponding to the absence of either the C8β or the C8α-γ subunit (6). The two types of deficiency are clinically indistinguishable. Seventy-three patients with C8 deficiency were reported in comprehensive reviews of the literature through 1990. (1, 2). The type of C8 deficiency was delineated in 58 patients: 48 (83%) were due to C8β deficiency and 10 (17%) were due to C8α-γ deficiency. Four additional C8α-γ deficient patients have been reported since 1990 (7, 8). Of the 14 C8α-γ deficient patients, 8 (57%) were African Americans, 4 (28.5%) were Japanese Asians, 1 was Hispanic and 1 was a Sephardic Jew. The genetic basis for C8α-γ deficiency has been defined for 2 of the Japanese Asian patients (8) but remains uncharacterized in the African American population. We analyzed the C8A gene in four unrelated African American patients and identified a G→A mutation in intron 6 of C8A as a predominant cause of C8α −γ deficiency in African Americans.

Materials and Methods

Patients

Four unrelated, C8α-γ deficient African Americans from different states within the U.S. and a normal individual participated in this investigation after signing University of Iowa IRB-approved consent forms. Three of the patients were referred for evaluation of infection; two with recurrent neisserial infection and one with pneumococcal bacteremia ([2]; C8 kindreds 39, 48 and 49). The fourth individual was diagnosed during evaluation for chronic urticaria and progressive spondyloarthritis (9). Serum from all patients had absent or very low total hemolytic complement activity (CH50). C8 deficiency was established by a combination of immuno-detection assays and functional assays for C8 in the sera from three individuals. CH50 activity in these sera was restored by the addition of C8. C8 deficiency was established in the fourth patient by immuno-detection (9). C8α-γ deficiency was established in the first three patients by restoration of CH50 activity after mixing their sera with C8β deficient sera, which contains functional C8α-γ, as reported previously (6). C8α-γ deficiency was established in the fourth patient by the genetic analysis reported herein.

SDS-PAGE and immunoblotting

Immunoblotting was completed for two deficient individuals. Purified human C8 (Quidel, San Diego, CA) and serum samples were heated and resolved by SDS-PAGE, under both

non-reducing and reducing conditions. The separated proteins were electrophoretically transferred to nitrocellulose and then Western blotted with specific polyclonal rabbit antiserum raised against C8α-γ (10).

mRNA isolation

Human fibroblasts were isolated from skin biopsies from the first three patients and a normal control. Fibroblasts were cultured in MEM alpha, supplemented with 10% fetal calf serum, penicillin and streptomycin. Total RNA was extracted by guanidinium isothiocyanate lysis, followed by CsCl density-gradient ultracentrifugation (11). Poly (A) RNA was isolated using Poly (A) TRACT (Promega Corp., Madison, WI).

Reverse Transcription and PCR

mRNA was transcribed, PCR amplified and C8α and C8γ cDNA sequencing completed for two deficient individuals. Reverse transcription was carried out, as described previously (10), using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA), mRNA (0.1–0.5 μg/ml), and oligo-dT (Pharmacia Inc., Piscataway, NJ) or oligonucleotide primers based on the reported cDNA sequences for C8α and C8γ. Specific regions spanning the coding sequences for C8α and γ were amplified using primer pairs designed from published sequences (12, 13). An informative primer pair amplified the 558 bp fragment of C8α cDNA between base pairs 918 and 1451 (5’ primer, 5’-TTATTGGTGGGTGTAGGTGTATCC-3′; 3′ primer, 5’-CAGGAACGGTATGTAATGGTGCTC-3′). PCR products were sequenced directly (14).

Genomic DNA isolation, amplification and sequencing

Genomic DNA was harvested from lysed fibroblasts or from peripheral white blood cells from patient four, RNased (Boehringer Mannheim Biologics, Indianapolis, IN) and proteinase K digested (Boehringer Mannheim Biologics, Indianapolis, IN) (15). Genomic sequencing of C8A was completed for all four patients. Genomic DNA was amplified using primers based on both the published C8A genomic sequence (16), and additional intron sequence from Gen Bank. The junction between intron 6 and exon 7 was amplified using primers: 5’-ATTACAGGCATGAGCCACTGC-3′ and 5’-TTTGCTTTGTCAATCACCAGG-3′. Amplified DNA was sequenced directly in our lab (S^{35} Sanger Sequencing) or at the Genomics Division of the Iowa Institute of Human Genetics (Big Dye Terminator chemistry – v3.1).

In Vitro splicing reactions

Genomic DNA, from one normal and one C8α-γ deficient individual, was amplified using the primer pair [5’: (5’GGGGAATTCATTACAGGCATGAGCCACTGC-3′); 3′ : (5’CCGCTCGAGTTTGCTTTGTCAATCACCAGG-3′) containing *EcoRI* and *XhoI* sites respectively. These primers define a 333 bp fragment extending from 106 bp upstream to 227 downstream of the 3′ splice site (SS) of exon 7. The amplified PCR product was sequenced, digested with *XhoI* and *EcoRI* (New England BioLabs Inc., Beverly, MA) and ligated into pHS1-X, which contains an HIV-1 cassette that can be transcribed *in vitro* (17). Plasmids were purified by CsCl and linearized by *BamHI*, 177bp downstream of the intron

6/exon 7 junction. Linearized DNA was transcribed *in vitro* by T3 RNA polymerase (Ambion, Austin TX) using radiolabeled ^{32}P-UTP, and added to splicing reactions incubated at 30ºC for 2 hrs. (17). Splicing reactions containing equal amounts of radioactivity were loaded onto denaturing polyacrylamide gels, run at 600V, dried and exposed to Hyperfilm-MP (Amersham Life Science).

Results

Sera from C8-deficient African-American patients lack the C8α-γ subunit

A previous study showed that sera from C8α-γ deficient patients contained low but detectable levels of a non-functional C8α-γ subunit (18). We looked for C8α-γ in sera from two deficient patients (Fig.1). When resolved on non-reducing gels, the positive controls (purified C8 and normal human sera) showed a reactive species migrating at 86 kDa, consistent with the presence of C8α-γ. In contrast, sera from the deficient patients, when probed with C8α-γ antiserum, contained no detectable immuno-reactive subunit of this molecular weight. When resolved under reducing conditions, the pooled normal human sera contained proteins migrating at 64 and 22 kDa (the molecular weights of C8α and γ respectively), but patient sera showed no similarly reactive species. Identical results were obtained when the sera were selectively precipitated with polyethylene glycol or ammonium sulfate (data not shown). We conclude that the sera from these African-American patients do not contain detectable levels of the C8A or C8G gene products, or do so at levels below the assay sensitivity.

Patients' C8α cDNA contains a 10-nucleotide insertion

To determine whether affected patients express mutant C8α or C8γ, the corresponding cDNAs were analyzed by RT-PCR. Sequencing confirmed that the C8γ coding region in the two patients examined was normal. In contrast, the sequence of C8α cDNA contained a ten base-pair (bp) insertion (5'-TTGCTGGCAG-3′), between nucleotides (nt) 992 and 993 (Fig. 2). Comparing the sequence of the PCR product to the C8A genomic sequence revealed that nt 992 and 993 mark the splice junction between exons 6 and 7, and that the 10 bp sequence was identical to the intronic sequence immediately upstream of the junction between intron 6 and exon 7 (16), suggesting the existence of an insertional splicing defect.

Affected patients share a mutation that creates a new 3′ splice site for exon 7

To determine why 10 bp are inserted at the boundary between intron 6/exon 7, we sequenced genomic DNA from normal and deficient patients. Six of the eight C8A alleles contained a G→A transition, twelve residues upstream of the intron 6/exon 7 junction. This analysis confirmed an exact match between the 10 bp insertion in the cDNA from deficient individuals and the 10 bp downstream of the mutation in their genomic DNA (Figs. 2, 3a). Two patients were found to be compound heterozygotes. The two C8A alleles in these patients were determined to be due to a previously described C → T transition at bp 1407 in exon 9 (supplemental Fig. 1) that creates a premature stop codon (8).

The shared G→A transition reported here converts a CGG in intron 6 to a CAG 12 nt upstream of the wild-type SS (Fig. 3b) thereby creating a new 3′ SS. Both the original and

the newly created CAG 3′ SS for C8A intron 6/exon 7 exactly match the consensus 3′ SS sequence (19). The data also suggest that the mutant 3′ SS is highly preferred *in vivo*, since no normal transcripts were detected in the RT/PCR sequences obtained from the C8α-γ deficient patients.

The upstream, mutant 3′ splice site is used exclusively in in vitro splicing reactions

To confirm that the mutation created a new and preferred 3′ consensus acceptor site for exon 7, we tested its function in an *in vitro* splicing system. Genomic DNA containing the C8 intron 6/exon 7 boundary was purified from normal and affected individuals (these sequences differed only in the G→A mutation). Amplified fragments were ligated into an *in vitro* transcription vector that contained an HIV-1 reporter, the resulting plasmids linearized and transcribed. The resulting RNA, composed of the heterologous HIV donor SS followed by 283 nucleotides encompassing the C8A intron 6/exon 7 boundary (Fig.4a), was tested in *in vitro* splicing reactions.

The results confirmed the point mutation creates a preferred 3′ SS (Fig. 4b). All unspliced transcripts (568 nt) co-migrated, as did the products from the first splicing step, which contain the lariat plus the C8 exon 7. In contrast, both the free intron (released during the second step of splicing) and the spliced exons differed depending on whether the RNA sequence was from normal or deficient subjects. In the latter case, the splicing products migrated as predicted if the G→A mutation at the −12 position had indeed created a new 3′ SS: the mutant transcript produced an excised intron that was slightly smaller (migrating at ~396 vs. ~406 nt) and a spliced exon that was slightly larger (migrating at ~308 vs. ~298 nt) compared to the wild-type samples. Moreover, although the mutant transcript still contained both 3′ splice sites, only the upstream mutant site was selected in the *in vitro* splicing reactions. Longer gel exposure did not reveal any mutant RNA transcripts spliced at the wild-type 3′ SS, suggesting the original 3′ SS is never used, or is used at a frequency below the level of detection.

Discussion

We explored the basis for C8α-γ deficiency in four unrelated African American individuals at the level of the secreted protein, mRNA and genomic DNA. These patients harbored the same mutation in at least one of their C8A alleles: a G→A point mutation 12 residues upstream of the intron 6/exon 7 boundary. This mutation converts a CGG to a CAG and in the process creates a preferred 3′ SS upstream of the normal intron6/exon 7 junction, causing exon 6 to be extended by 10 nt at the 5’ end. This 10-nt insertion shifts the reading frame and creates a stop codon at bases 1050–1052 (57 nt downstream from the mutant 3′ SS). This finding is consistent with the failure to synthesize C8α or the synthesis of a dysfunctional protein. Either mechanism would account for the absent hemolytic activity in the sera from these individuals and contribute to their risk of infection or auto-immune disease. If translated, the mutant transcript would be predicted to give rise to a 304 amino acid protein of 34 kDa (~ 55% the size of the normal C8α). If this truncated form were synthesized, it might be able to form a covalent bridge with C8γ through cysteines 164 and 40 in the respective proteins (5). We were unable to detect either a smaller C8α-γ subunit or

a truncated C8α protein product in the deficient sera, suggesting either that the aberrantly spliced transcript is subject to nonsense-mediated decay (20) or that a truncated protein might be unstable and quickly degraded.

While disease-associated mutations that cause splicing defects are common, most either destroy the original site or activate cryptic sites; mutations creating a new and preferred SS are uncommon. A review of point mutations in the vicinity of mRNA splice junctions reported that 13 of 101 mutations generated a new SS. Of these, only six created a new consensus 3′ SS, and just three showed evidence that the novel spice site was used (21). Of these, the aberrant splicing of phenylalanine hydroxylase mRNA is most similar to the one described here. In the case of phenylalanine hydroxylase deficiency, a G→A exchange at the −11 position in intron 10 causes a nine-base insertion in the mRNA that, although in frame nevertheless destroys enzyme function (22). Thus, the molecular basis for C8α-γ deficiency in African Americans defined here is unique.

Structural features in pre-mRNA that affect recognition of a 3′ SS include the nucleotide immediately preceding the consensus AG, the proximity to the branch point, and the strength of the upstream polypyrimidine tract (23–25). For both the wild-type and mutant acceptor sites reported here, the nucleotide immediately preceding the consensus AG is a C, the most favorable from the standpoint of a 3′ SS consensus sequence (19). The *in vitro* splicing experiments demonstrate that mRNA substrates derived from normal or deficient persons are functional, implying both splice sites are favorably situated with respect to any cis-acting elements that influence recognition of the wild-type site. In spite of this equivalence and the presence of both site splice sites in the mRNA from the C8α-γ deficient individuals, the new 3′ SS created by the G→A mutation in these individuals is by far preferred.

One possible factor driving selection of the mutant SS is the relative strength of the polypyrimidine tract. The most important determinants of polypyrimidine tract strength are the proportion of uridine (thymidines in sequencing reactions) residues in the 12-nt stretch preceding the CAG and the number of those residues that are consecutive (23–25). In the normal gene, those 12-nucleotides contain 5 thymidines, of which just two are consecutive. In contrast, the 12 nucleotides preceding the consensus 3′ SS in the mutated gene contain 6 thymidines, all but one of which are consecutive (Fig. 3c). This difference is even more pronounced if the comparison is extended further upstream. Thus, in C8 α-γ-deficient individuals, the point mutation in C8A intron 6 creates a new 3′ SS with a substantially stronger polypyrimidine tract, which likely accounts for its highly preferred utilization.

The molecular basis for C8α-γ deficiency reported here differs from that reported in two unrelated Japanese persons (8). In those patients, three of the four null alleles involved a point mutation at the second exon-intron boundary which inactivated the conserved 5’ SS and presumably leads to exon skipping. The fourth allele involved a C→T transition that generated a premature stop codon. Thus, two of the three distinct C8A null alleles described to date involve splicing defects. In contrast, in four of the seven described null alleles in the highly homologous C8B gene, unique C→T transitions produced premature stop codons; none of the remaining three null alleles was caused by a splicing defect (26, 27).

Our findings should be reconciled with those of Tedesco *et al.* (18) showing sera from deficient patients contain minute quantities (≤ 0.5%) of a normal-sized C8α-γ subunit, which lacked functional activity in hemolytic overlay agarose gels (18). One explanation for this discrepancy is that the molecular basis for the deficiencies may differ in persons from different ethnic backgrounds, as described above. Alternatively, splicing reactions *in vitro* and *in vivo* are distinctly different; *in vitro* the substrate is a pre-synthesized transcript, while *in vivo* splicing is co-transcriptional. Therefore, splicing *in vivo* might allow recognition of the wild-type site, albeit at a very low frequency. However, if this were the case, one would expect the synthesized C8α-γ to possess normal hemolytic activity. The inability of Tedesco *et al.* to detect hemolytic activity associated with the minute amount of C8α-γ subunit (18) might reflect the different sensitivities of the functional and immuno-assay systems. Lastly, neither the molecular basis for C8α-γ deficiency described here nor that described by Kojima (8) sheds light on the mechanism underlying the reduced amount of C8β in these sera, other than to indicate it is not due to a direct effect of these genetic alterations on C8β.

In summary, we identified a G→A mutation in intron 6 of C8A as a predominant cause of C8α−γ deficiency in African Americans. This mutation creates a new and highly preferred 3′ SS, results in a 10-bp insertion, shifts the reading frame and produces a downstream premature stop codon.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the excellent editorial assistance provided by Drs. Diana Colgan and John P. Atkinson, and the supportive contribution of referring physicians Drs. Jerry A. Winkelstein, Mary Beth Fasano, James E. Peacock, David S. Stephens, Edward F. Hendershot, Carlos Del Rio, and Dai Park.

Big Dye Terminator sequencing data presented herein were obtained at the Genomics Division of the Iowa Institute of Human Genetics which is supported, in part, by the University of Iowa Carver College of Medicine.

This work was supported by a Merit Review Award from the Department of Veteran's Affairs (PD) and by PHS grants CA28051 from the National Cancer Institute and AI36073from the National Institute of Allergy and Infectious Diseases (both to CMS)

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KEY POINTS

- C8α−γ deficient African Americans share a G→A mutation in intron 6 of the C8A gene.
- This change creates a new, preferred 3′ splice site causing a 10 nt mRNA insertion.
- The insertion shifts the reading frame producing a premature stop codon downstream.

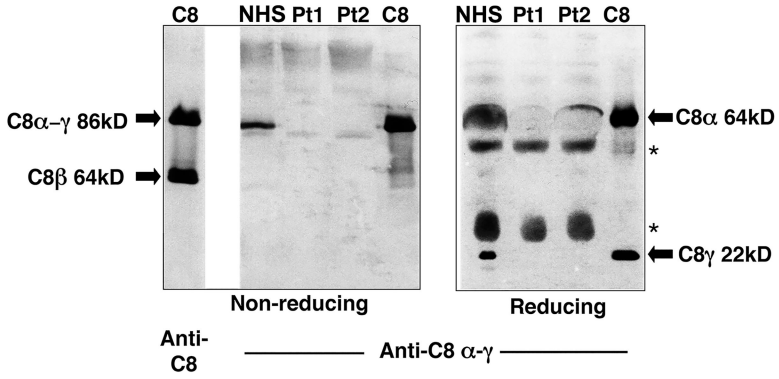

FIGURE 1.

Immunoblots comparing normal and C8 α-γ deficient sera. Western blots of C8 or patient sera were probed using rabbit polyclonal antibodies raised against C8 or C8 α-γ and developed with alkaline phosphatase labelled goat anti-rabbit IgG. Left Panel: non-reducing conditions. The position of the C8α−γ and β subunits is shown in the far-left lane. Right Panel: reducing conditions. The position of the individual C8α and C8γ polypeptide chains is shown in the far-right lane. In contrast to their presence in normal human sera (NHS), neither the C8α-γ subunit, nor the individual α and γ polypeptides was evident in the sera from C8α-γ deficient patients (Pt). Each patient's serum was examined at least twice. In reducing gels, the bands (*) at ~55 and 25 kDa are consistent with the heavy and light chains of IgG.

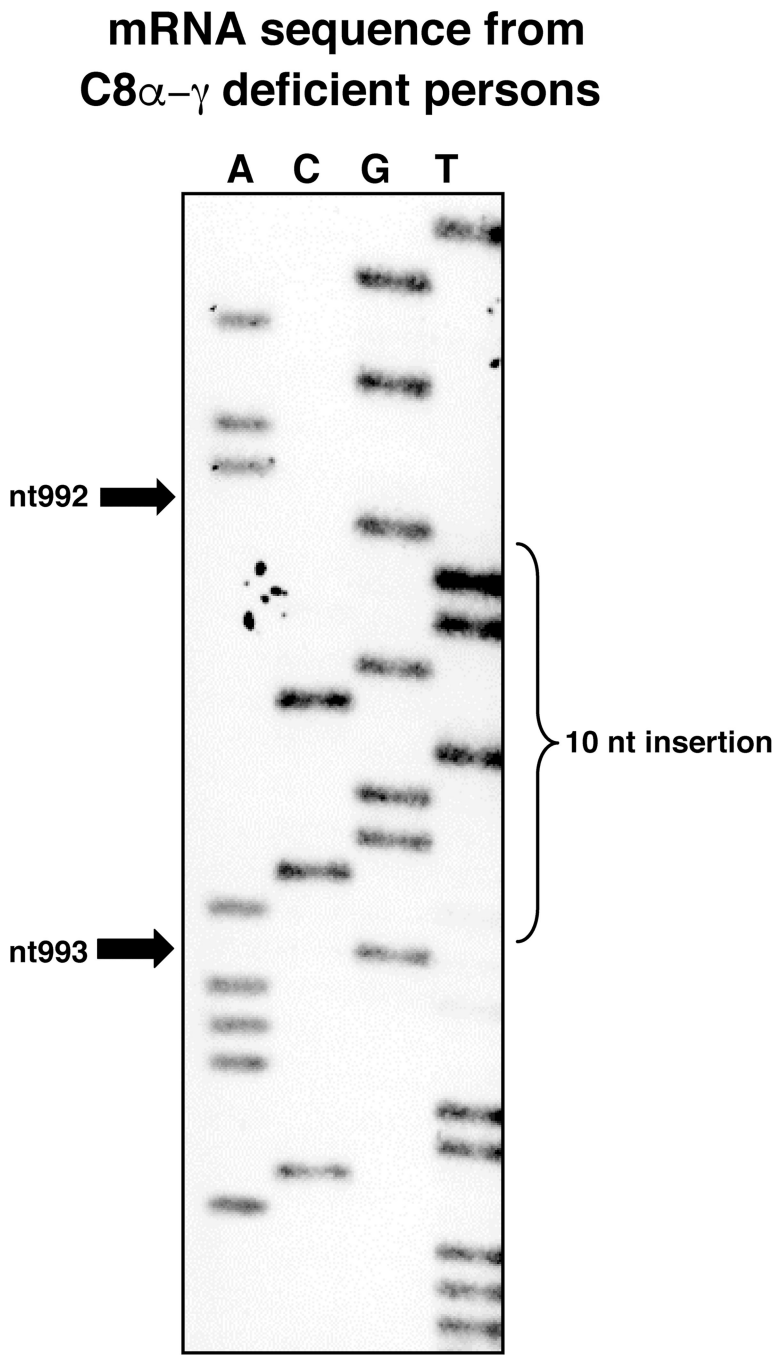

FIGURE 2.

C8α-γ-deficient Patients' C8α cDNA contains a 10 base pair insertion. C8α cDNA, generated from C8α-γ-deficient individuals' mRNA was sequenced directly. Comparing this sequence to that reported previously (11, 15) revealed a 10 bp insertion between nt 992 and 993. Each patient's cDNA was sequenced at least twice.

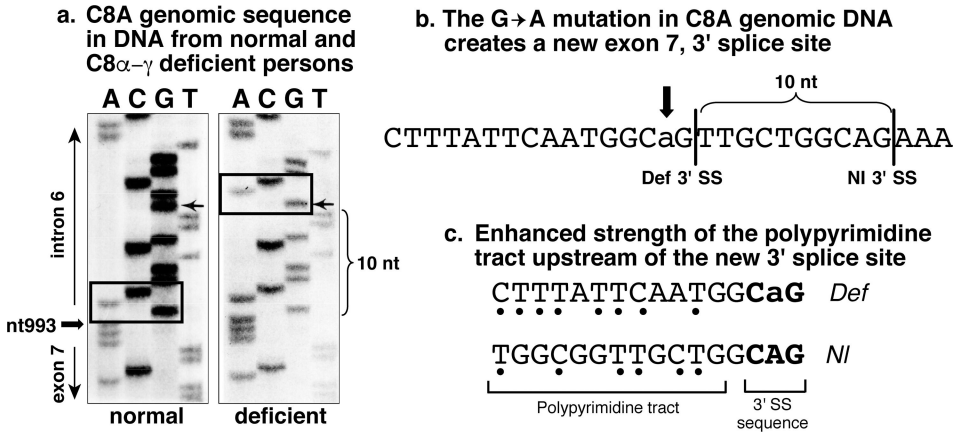

FIGURE 3.

Comparison of genomic sequences from normal (Nl) and C8α-γ deficient (Def) persons. *a)* DNA was extracted from normal and patient fibroblasts, amplified by PCR, and sequenced. Sequencing revealed a G→A mutation (arrows) 12 bp upstream from the start of exon 7, at nt 993. This mutation creates a new consensus 3′ SS (box) in the intronic sequence in the DNA from deficient individuals that is upstream of the usual 3′ SS (box) in DNA from normal individuals. Amplified genomic DNA from each patient was sequenced at least twice. *b)* The G→A mutation creates a new consensus 3′ SS upstream of the intron 6/exon 7 boundary in DNA from the normal individual. The vertical arrow indicates the G→A point mutation, which is noted by a lower case "a." The newly created 3′ SS signal sequence and the one found in normal individuals are designated by the broad vertical bars. A bracket encompasses the 10 nt of intronic sequence that becomes included in exon 7 when the newly created 3′ SS is used. *c)* The newly created, putative 3′ SS has enhanced polypyrimidine tract strength. The DNA sequence upstream of the 3′ SS of C8A exon 7 selected in individuals who are deficient (upper sequence) or normal (lower sequence) are aligned to facilitate comparison of their polypyrimidine tracts. The point mutation in deficient individuals is in lower case; 3′ SS consensus is in bold; and pyrimidines in the polypyrimidine tract are indicated by black circles below the nucleotide. The DNA sequence upstream of the 3′ SS of C8A exon 7 in normal individuals contains fewer pyrimidines (6, vs 8, respectively) and fewer of these occur as contiguous nucleotides (4 of 6 vs 7 of 8, respectively) than the analogous sequence in the DNA from deficient persons (see the discussion section for the relevance of these changes).

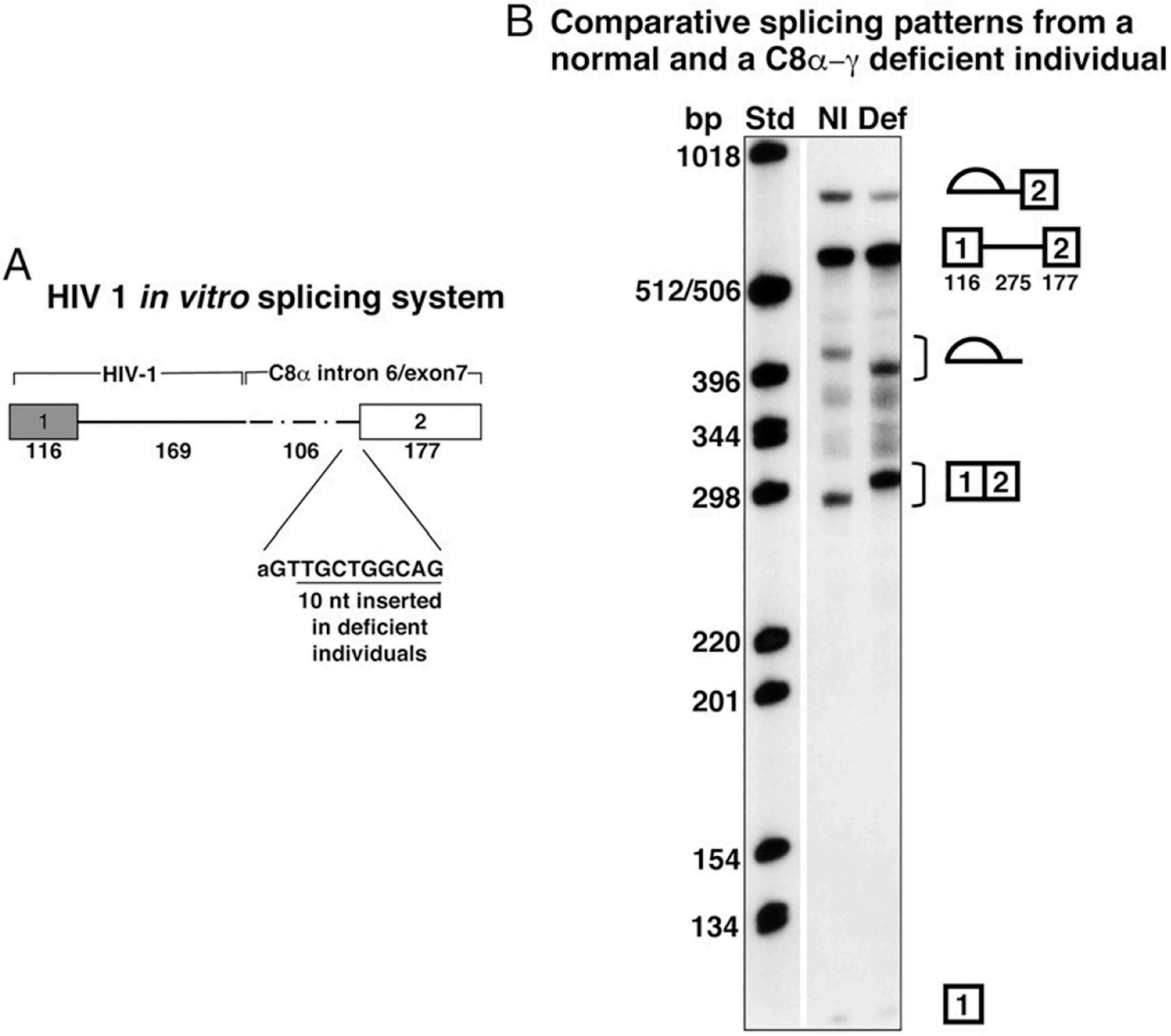

FIGURE 4.

The newly created SS is highly preferred. (A) Schematic of the RNA splicing substrates, which contain the heterologous HIV 116 nt 59 exon (box 1), 169 nt intervening sequence followed by 106 nt from the 39 end of C8A intron 6, and 177 nt from the 59 end of C8A exon 7 (box 2). (B) DNA m.w. standards (Std) and their respective lengths (in bp) are shown in the left lane. Intermediates and products of splicing reactions containing either normal (Nl) or deficient (Def) RNA substrates are shown in the right two lanes, respectively. The vertical white line denotes that the standard and the experimental lanes were separated in the original gel and later assembled to create a composite figure from the corresponding autoradiograph. The diagram at the right depicts spliced products and intermediates [compare with (A)]. Substrates derived from a C8α-γ-deficient patient sequence are consistent with the G→A mutation at the 212 position, creating a new 39 SS. The mutant

transcript produced a slightly smaller excised lariat (~396 versus ~406 nt) and a slightly larger spliced product (~308 versus ~298 nt) compared with the normal intron and spliced products. Lariat products migrate with slower mobility than linear RNA of the same size. Splicing reactions were performed once as described in the Materials and Methods.