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Genetics

Clusters of intragenic *Alu* repeats predispose the human C1 inhibitor locus to deleterious rearrangements

(genetic disease/hereditary angioedema/serine protease inhibitor/complement)

DOMINIQUE STOPPA-LYONNET*, PHILIP E. CARTER†, TOMMASO MEO*, AND MARIO TOSI*‡

*Unité d'Immunogénétique and Institut National de la Santé et de la Recherche Médicale, U. 276, Institut Pasteur, Paris, France; and †Department of Biochemistry, University of Aberdeen, Aberdeen, United Kingdom

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ABSTRACT **Frequent alterations in the structure of the complement component C1 inhibitor gene have been found in patients affected by the common variant of hereditary angioedema, characterized by low plasma levels of C1 inhibitor. This control protein limits the enzymic activity of the first component of complement and of other plasma serine proteases. Sequence comparisons of a 4.6-kilobase-long segment of the normal gene and the corresponding gene segments isolated from two patients carrying family-specific DNA deletions point to unusually long clusters of tandem repeats of the *Alu* sequence family as a source of genetic instability in this locus. Unequal crossovers, in a variety of registers, among *Alu* sequences of the clusters result in deletions of variable length that encompass exon 4. In a third family, exon 4 was instead found to be duplicated along with the same tracts of flanking introns lost in one of the deletions. In addition to undergoing *Alu*-mediated partial deletions and duplications, the gene is also a target for more recent retroposition events. Gross alterations in the C1 inhibitor gene account for about 20% of the hereditary angioedema chromosomes and consequently make this gene a prime example of the mutagenic liability of *Alu* repeats.**

The serine protease inhibitor complement component C1 inhibitor (C1 INH) plays a key role in the control of the classical pathway of complement activation, since it is the only inhibitor of the enzymic subcomponents C1r and C1s of C1, the first complement component (1). Furthermore, C1 INH also inactivates the serine proteases kallikrein, plasmin, and coagulation factors XIa and XIIa (reviewed in ref. 2).

The most severe consequence of C1 INH deficiency, be it due to impaired biosynthesis or to the presence of a dysfunctional form, is uncontrolled complement component C1 activity with subsequent excessive cleavage of C2, the second complement component (2). A C2-derived peptide, recently shown to enhance vascular permeability (3), is believed to be the main inducer of the swelling attacks typical of angioedema (4). The hereditary form of angioedema (HAE) is a disease transmitted as an autosomal dominant trait (5, 6). Defects in the reactive site of the C1 INH protein, recently documented in molecular terms (7, 8), often explain the forms of the disease in which a dysfunctional protein, which can sometimes accumulate to levels even higher than normal is synthesized (type II HAE). In contrast, little is known about the molecular nature of the gene defects that are responsible for the more common form of HAE (type I), characterized by low serum levels of normal C1 INH protein (6).

Previous studies of type I HAE families by using molecular probes for the C1 INH gene allowed us to show that DNA length variations in this locus cosegregate with the disease. The detection of family-specific sets of restriction fragments led us to postulate that distinct alterations cluster within the 5′ half of the gene§ in a significant proportion of type I families (9). Here we describe a "hot spot" of genetic alterations, related to unusual *Alu* repeat clusters, which accounts for the occurrence of the disease in a significant fraction of families.

EXPERIMENTAL PROCEDURES

We examined gene alterations characteristic of four unrelated kindreds affected by type I HAE (families F1, F3, F4, and F5). The disease-related restriction fragments found in families F1, F3, and F4 have been described (9).

DNA Blot-Hybridization Analysis. The probes used were either the entire insert of plasmid pHC1-INH/1 (10) or a *Pst* I fragment thereof. An exon 4-specific probe was produced by polymerase chain reaction (11) using a full-length cDNA clone kindly provided by S. Bock (12). DNA fragments were radioactively labeled by using a random priming protocol (13).

Cloning and Characterization of Defective Genes. Genomic libraries were constructed from the DNA of member IV2 of family F1 and from a patient of family F4, both reported on previously (9), by using the bacteriophage λ FIX vector (Stratagene). About one million phages from each library were plated on *Escherichia coli* strain C600 and screened with the exon 4- to 8-specific cDNA probe. Four positive clones were purified from each library and analyzed by double digestion with the enzymes *Sal* I and *Bam*HI.

Sequencing Strategies. A detailed restriction map of the portion of the normal C1 INH gene extending from the *Bam*HI site in exon 3 to the 3′ boundary of exon 6 (see Fig. 2) was established by using subcloned fragments of cosmid clone 6d (14). To define the boundaries of the family-specific deletions found in families F1 and F4, appropriate fragments, outlined in Fig. 2, were sequenced in M13 vectors by using single-strand (15) or double-strand (16) deletion protocols and the modified dideoxy method (17).

RESULTS

Family-Specific Deletions. DNA blot analyses of HAE type I kindreds showed that partial C1 INH gene rearrangements are probably the single most frequent class of genetic lesions in the pathogenesis of HAE (9). To localize more precisely such structural alterations, we studied the DNA of affected members of unrelated families by using the enzyme *Bam*HI and overlapping hybridization probes. Fig. 1 demonstrates that several family-specific changes occur within the 6.9-kilobase (kb)-long *Bam*HI fragment comprising exons 3–6. As expected from the dominant inheritance of the disease,

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Abbreviations: C1 INH, complement component C1 inhibitor; HAE, hereditary angioedema; IVS, intervening sequence (intron).

‡To whom reprint requests should be addressed.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M30688).

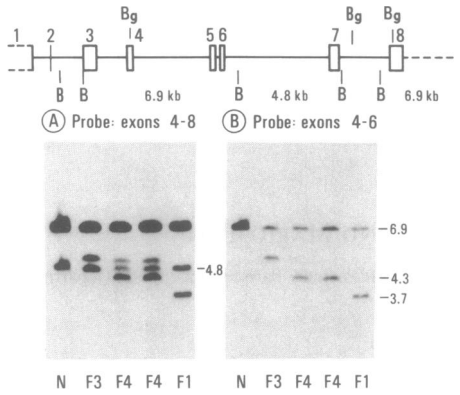

FIG. 1. DNA blot analysis of deletions in the 5′ half of the C1 INH gene. Leukocyte DNA was digested with the *Bam*HI endonuclease, and the blot was hybridized with a probe covering most of the gene (*A*) or with a probe specific for exons 4–6 (*B*). The autoradiogram compares the hybridization of a control DNA (N), of DNA from unrelated affected persons (F1 and F3), and of DNA from affected mother and daughter from a different family (F4). Note that in *A* the 6.9-kb band represents a doublet consisting of the fragment that contains exons 3–6 and of the 3′ terminal *Bam*HI fragment. The diagram of the exon–intron structure and the restriction map are reproduced from ref. 14. Exon 1 is depicted as an open rectangle to indicate that the 5′ end of the gene is not yet mapped precisely. Only the *Bam*HI (B) and *Bgl* II (Bg) restriction sites are shown.

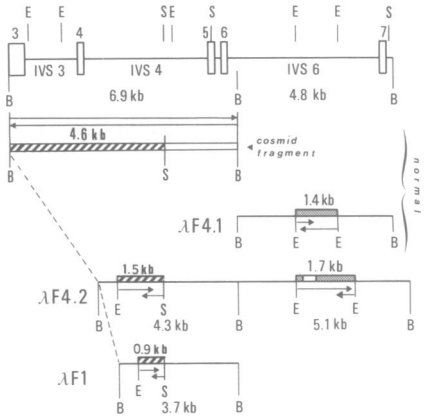

FIG. 2. Isolation of normal and altered restriction fragments. The normal 6.9-kb *Bam*HI fragment spanning exons 3–6 (rectangle marked "cosmid fragment"), derived from cosmid clone 6d (14), was subcloned and sequenced on both strands. The normal 4.8-kb *Bam*HI fragment covering intron IVS 6 and exon 7 was isolated from clone λF4.1, which represents the normal chromosome of the F4 patient. Its central, stippled *Eco*RI fragment was sequenced as indicated by the arrows. To define the boundaries of the deletions observed in families F1 and F4, the 3.7- and 4.3-kb-long *Bam*HI fragments, derived from clones λF1 and λF4.2, respectively, were compared by restriction analysis to the corresponding normal 6.9-kb *Bam*HI fragment. The hatched 0.9- and 1.5-kb *Eco*RI–*Sac* I fragments, which encompass the boundaries of the deletions in families F1 and F4, respectively, were sequenced and compared with the corresponding normal sequence (4.6-kb-long hatched portion of the normal 6.9-kb *Bam*HI fragment). The stippled 1.7-kb *Eco*RI fragment of clone λF4.2 harbors an insertion of about 300 base pairs (open rectangle), whose length is consistent with the presence of an additional integral *Alu* repeat (see Fig. 6). B, *Bam*HI; E, *Eco*RI; S, *Sac* I.

the 6.9-kb hybridization band displays a reduced intensity in patients' DNA, consistent with their heterozygous genotype. This quantitative difference is more prominent when the shorter probe, specific for exons 4–6, is used (Fig. 1*B*). In addition, each patient carries a shortened and family-specific variant of the 6.9-kb-long *Bam*HI fragment.

The larger probe, specific for exons 4–8 (Fig. 1*A*), reveals a 4.8-kb-long *Bam*HI fragment common to normal individuals and patients, which however displays a reduced intensity in the two members of family F4. Presence in the latter family of an additional hybridization band of slightly larger size but of the same intensity suggests that these patients carry a chromosome with an allelic form of the 4.8-kb *Bam*HI fragment. The apparent cosegregation of the changes that distinguish family F4 indicates that both have occurred on the same chromosome.

Additional DNA blot analyses (data not shown) enable us to conclude that the size of the deletion in family F3 is larger than could be inferred from the size of the variant *Bam*HI fragment shown in Fig. 1 and that in fact it includes exon 3 and its exonic *Bam*HI site. The deletions observed in families F1 and F4, on the other hand, although of a different size, appear to fall entirely within the boundaries of the 6.9-kb *Bam*HI fragment that carries exons 3–6. We therefore set out to compare, by cloning and nucleotide sequence analysis, this normal *Bam*HI fragment with the deleted variants found in families F1 and F4. The normal 6.9-kb-long fragment was isolated from a previously described C1 INH cosmid clone (14), whereas the deleted gene segments were cloned from bacteriophage λ libraries representing families F1 and F4. In the case of family F1, four C1 INH clones were isolated, one of which contained the 3.7-kb-long deleted *Bam*HI fragment (Fig. 1 and fragment λF1 in Fig. 2). Four overlapping clones were isolated from the F4 library, two of which carried a normal 4.8-kb-long *Bam*HI fragment (see fragment λF4.1 in Fig. 2) and two others both containing the altered restriction fragments (see the 4.3- and 5.1-kb-long *Bam*HI fragments carried by clone λF4.2 in Fig. 2).

The complete nucleotide sequence of the normal 6.9-kb-long *Bam*HI fragment was established, and the portion most relevant to the reconstruction of the deletion mutations observed in families F1 and F4 is shown in Fig. 3. A remarkable feature of the normal sequence is the presence of multiple tandemly arranged repeats of the *Alu* sequence family around exon 4. Indeed most of intron 3 (IVS 3) consists of three direct *Alu* repeats, whereas seven additional repeats are found in intron IVS 4 (Fig. 4). The boundaries of the family-specific deletions found in the F1 and the F4 kindred are delimited in Fig. 3 by boxed sequences. The breakpoints of the deletion in family F1 were found within *Alu* sequence 1 and *Alu* sequence 8, respectively. Although each element in this cluster can be individually recognized because of sequence divergence, amounting to about 20% such as is generally found for *Alu* sequences (18), the intrafamily sequence conservation precludes definition at the nucleotide level of the deletion breakpoints. However, these can be confined to within identical 14-base-pair-long stretches boxed within *Alu* sequence 1 and *Alu* sequence 8. Likewise, the deletion breakpoints found in the corresponding area of the defective gene of family F4 fall within *Alu* repeats 1 and 6, respectively, and can be narrowed down to identical stretches of 26 nucleotides (boxed and marked F4 in Fig. 3). These sequence comparisons suggest that the genetic lesion common to families F1 and F4 is the loss of exon 4, although the extent of the deletion differs, since the 3′ breakpoints involve different *Alu* elements. One should notice moreover that the 5′ breakpoints of these deletions also differ, even though they are located within the same *Alu* element.

Unequal Recombinations Generate Deletions and Duplications. Unequal crossovers at meiosis, mediated by mispaired *Alu* sequences, is the most likely explanation for the recurring deletions of exon 4 and their length variation, as depicted in Fig. 4. Recombination within *Alu* elements 1 and 8 yields the more extensive (family F1) of the two deletions described here. In a reciprocal fashion, duplications of a DNA segment comprising exon 4 should also occur, as suggested in Fig. 4*B*.

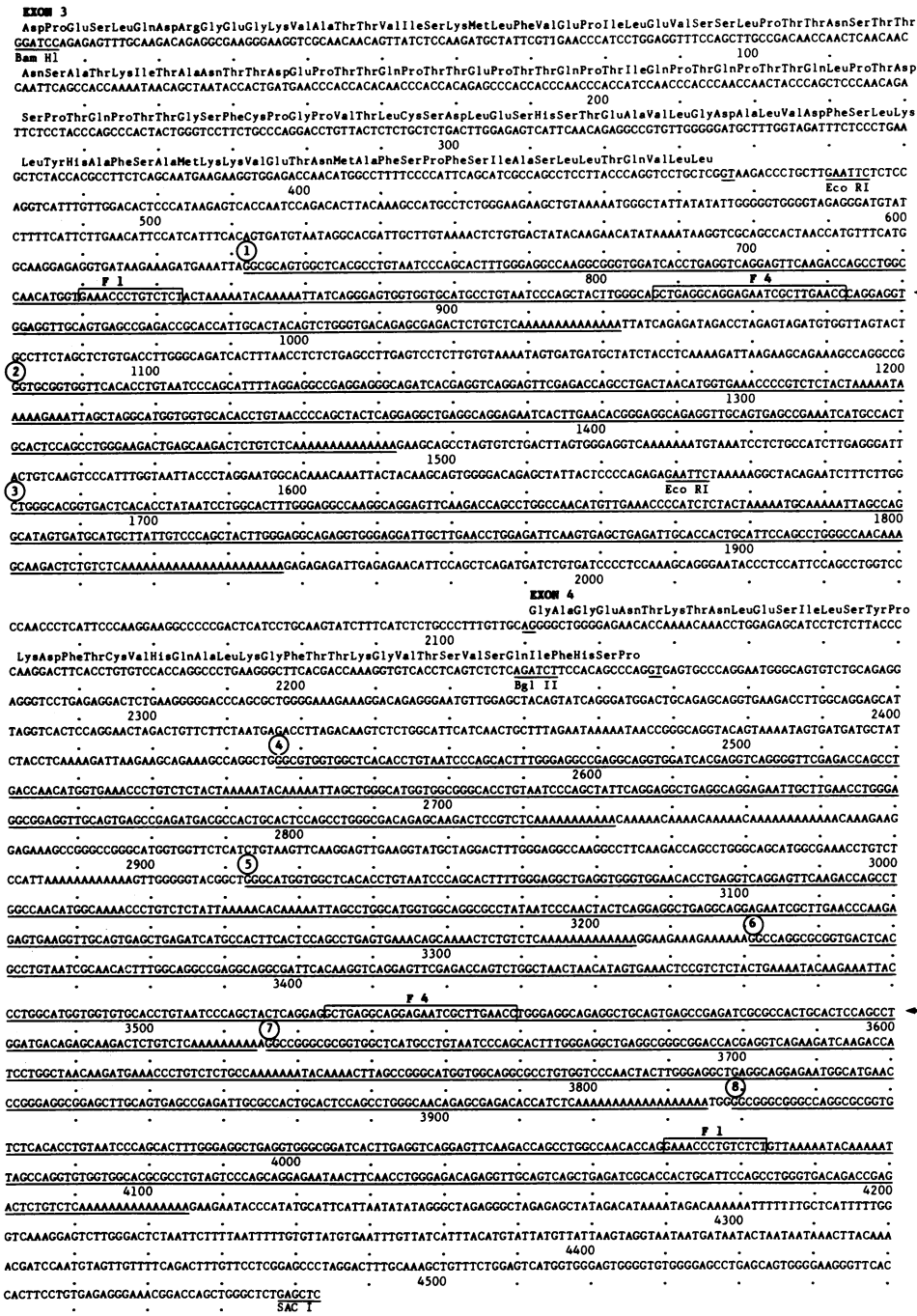

FIG. 3. Sequence analysis of the boundaries of deletions δ1 and δ4 found in families F1 and F4, respectively. Nucleotide sequences deduced from the 0.9- and 1.5-kb-long *Eco*RI–*Sac* I fragments, respectively, were compared to the sequence of the 4.6-kb-long segment of the normal gene that extends from the *Bam*HI site in exon 3 to the *Sac* I site in intron IVS 4 (see Fig. 2). The boundaries of the δ1 and the δ4 deletions fall within the repeated sequences boxed and labeled F1 and F4, respectively. In addition to these deletions, the only allelic differences found were a G → T and a G → A substitution, both in family F1 at positions 4515 and 4522, respectively. Note the tandem array of *Alu* elements, underlined and marked with circled numbers 1–8. Additional *Alu* repeats, not shown in this sequence, are located in the more 3′ portion of intron IVS 4 (see Fig. 4).

Chromosomes carrying such duplications may even be expected among HAE patients, if one postulates a deleterious effect on gene expression ensuing from exon 4 duplication or from alterations in the surrounding introns. In fact, in an extended survey of type I HAE families, we did find a patient featuring a partial duplication of the C1 INH gene similar to the one depicted in Fig. 4*B*. Analysis of the C1 INH locus of this patient (Fig. 5) was performed by using the enzymes *Bam*HI, *Bgl* II, and *Eco*RI and a probe specific for exon 4. The additional band at about 10 kb in the patient's *Bam*HI lane is already indicative of a duplication extending over a few kilobases and comprising exon 4, since the hybridization intensity of this patient-specific *Bam*HI fragment is about twice that observed for the normal 6.9-kb-long allelic fragment. Digestions with *Bgl* II, a marker of exon 4, allow one to estimate more precisely the extent of the duplication, as they reveal a patient-specific fragment of 3.2 kb, in addition to the normal 10-kb-long fragment that extends into the 5′ flanking region (14). Consistent with the heterozygous genotype of the patient, this 3.2-kb-long duplication-specific *Bgl* II band is

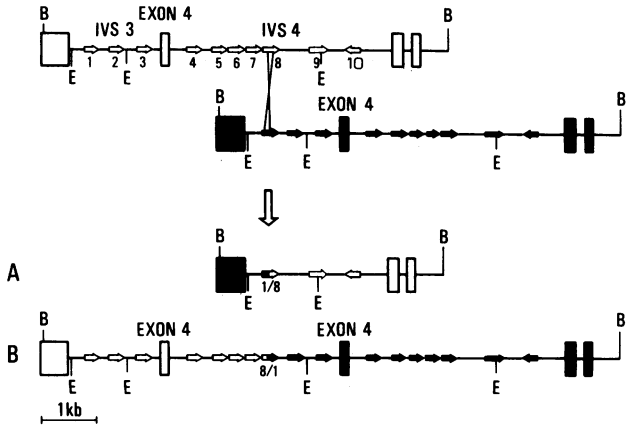

FIG. 4. Model of the observed deletions, based on *Alu*-mediated unequal crossovers. Unequal recombination between normal C1 INH genes exchanges out-of-register gene segments harboring exons 3–6. Tandemly oriented *Alu* sequences are denoted by arrows and are numbered 1–9. Note that a 10th *Alu* element is in the opposite orientation. (*A*) Deletion around exon 4 as observed in family F1. (*B*) Partial duplication postulated to occur by recombination within the first and eighth repeat. B, *Bam*HI; E, *Eco*RI.

weaker than the 2.23-kb band resulting from *Bgl* II/*Bam*HI double digestion, as the latter is present in both the normal and the partially duplicated gene copy. Moreover, an *Eco*RI recognition site, located between the second and third tandem *Alu* repeats (see Figs. 3 and 4), allows the portion of the intron IVS 3 contained within the duplication to be delimited. The breakpoint must lie 5′ to this *Eco*RI site. Indeed the patient-specific *Eco*RI fragment has precisely the same length as the patient-specific *Bgl* II fragment, and both allow the identification of a duplication unit of 3.2 kb. Consistent with the model depicted

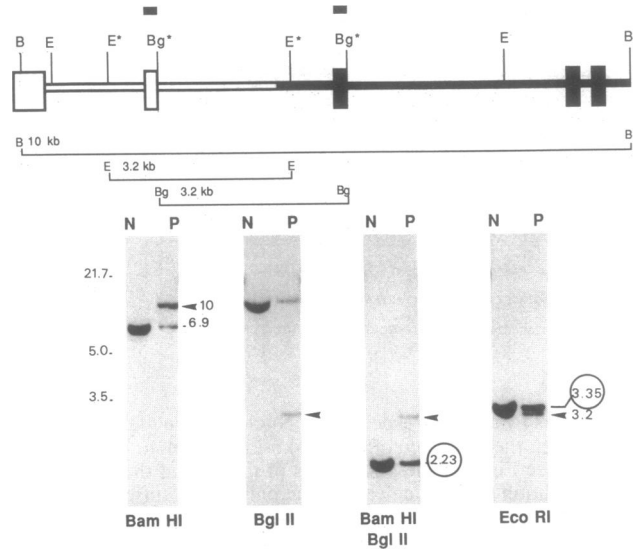

FIG. 5. Exon 4 is duplicated in family F5. This DNA blot analysis demonstrates a partial duplication that corresponds to the model shown in Fig. 4*B*. A restriction map of this duplication was constructed by comparing fragments detected with an exon 4-specific probe, which recognizes the short stretches shown as solid bars. Lanes carry restriction digests of patient DNA (lanes P) or of normal controls (lanes N). The diagram depicts the postulated recombination between C1 INH genes according to the model of Fig. 4*B*. Sites *Eco*RI (E) and *Bgl* II (Bg) that are marked by asterisks are duplicated, and the length of the duplication unit (3.2 kb) was deduced from the size of the patient-specific *Eco*RI and *Bgl* II fragments. The size of normal restriction fragments whose nucleotide sequence has been determined is marked by circles. B, *Bam*HI.

in Fig. 4*B*, the observed length of the duplication unit thus implies that *Alu* sequences 1 and 8 are most probably involved not only in the F1 deletion but also in the recombination event that gave rise to this duplication.

A Newly Inserted *Alu* Sequence in the C1 INH Gene. In addition to the excision of DNA around exon 4, the affected gene of family F4 also carries an apparent insertion in intron IVS 6 (see Fig. 2), which has led to the normal 4.8-kb-long *Bam*HI fragment being replaced by a 5.1-kb-long fragment. As this difference is fully accounted for by the size of the inner *Eco*RI fragments (Fig. 2), we compared their nucleotide sequences. Fig. 6 shows schematically that three *Alu* elements are located in the region of the normal *Eco*RI fragment and that the affected gene contains an additional *Alu* element. The latter is inserted precisely within the adenosine-rich sequence that defines the boundary between the similar left and right modules that make up the *Alu* elements (19). The sequence comparisons shown in Fig. 6 reveal that this newly inserted *Alu* element has some of the properties of retrotransposed DNA—namely, (*i*) duplication of the target sequence and (*ii*) presence at the 3′ extremity of a long adenosine-rich stretch.

DISCUSSION

Alu repeats are evolutionarily related to a functional gene encoding cytoplasmic 7SL RNA (20), of which they have also

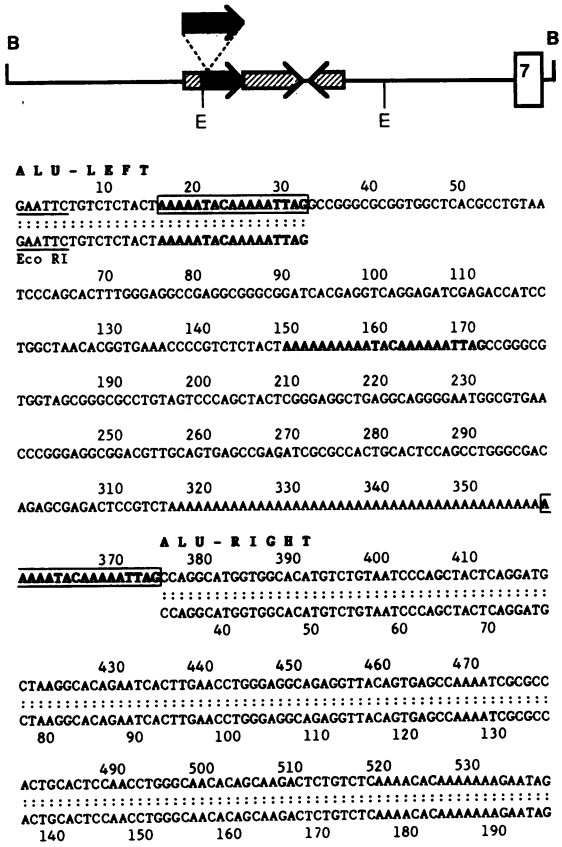

FIG. 6. Insertion of an additional *Alu* element within intron IVS 6. The *Eco*RI (E) fragment in the center of intron IVS 6 (see Fig. 2) is larger than normal in the affected gene of family F4 because of the presence of an additional *Alu* element. The nucleotide sequences correspond to the portions of *Alu* repeats indicated as full arrows. The insertion point of the additional *Alu* repeat is within the adenosine-rich segment (boldface letters) at the junction of left and right *Alu* monomers. This target sequence has been duplicated (boxed boldface letters). Note the long poly(A) stretch at the 3′ end of the inserted element. The shorter arrow denotes an incomplete *Alu* element consisting of a right monomer in the opposite orientation.

retained the potential to be transcribed by RNA polymerase III (21, 22).

That recombination between *Alu* repeats can be a frequent cause of gene deletion has been suggested by previous studies on familial hypercholesterolemia. The low density lipoprotein receptor gene in fact harbors multiple *Alu* repeats within several introns, as well as in the 3′ noncoding portion of its last exon (23). Moreover, a duplication of a large 5′ segment of the low density lipoprotein receptor gene also arose from unequal crossing-over between *Alu* sequences (24). These and other studies (25) suggested that the recombination breakpoints occur more frequently within the 5′ halves of *Alu* repeats, where the putative bipartite RNA polymerase III promoter is located. The breakpoints observed in families F1 and F4, however, depart from this trend. Long clusters of intragenic *Alu* repeats have also been detected in a few other cases, such as the human β-tubulin gene, which harbors 10 repeats within a single intron (26) and the human gastrin gene, in which an exon is flanked by a total of 8 repeat elements (27). However, in these cases, hereditary deficiencies have not been described. Hence, in the absence of the enrichment factor provided by disease sampling, the overall effect of *Alu* clusters on the stability of these genes can hardly be assessed.

We have also found an unexpected *Alu* repeat inserted into a preexisting *Alu* cluster of the C1 INH gene (family F4, Fig. 6). The association on the same chromosome of *Alu* transposition and an *Alu*-mediated deletion is intriguing. Pending a better estimate of the variability in the number of *Alu* repeats within the C1 INH gene, one should consider the possibility that the two alterations did not occur independently and more specifically that the insertion of an additional *Alu* element occasioned the misalignment that led to an unequal crossing-over. This additional *Alu* intrusion features the long poly(A) tail and the duplication of a short target sequence, which are hallmarks of genetic mobile elements subject to retrotransposition (22). Although such insertions seem to occur more frequently within the adenosine-rich 3′ flanking regions of *Alu* repeats (28), at least one other example of insertion within the junction between the left and right *Alu* monomer has been reported in the case of a human pseudogene encoding U2 small nuclear RNA (29). Closer inspection of the additional *Alu* repeat in the altered gene of family F4 revealed that its sequence complies with the consensus of the evolutionarily most recent *Alu* branch [i.e., the class IV of Britten *et al.* (30) or the subfamily Sb of Jurka and Smith (31)]. More generally, the 12 complete *Alu* repeats of the normal C1 INH gene described here (Figs. 4 and 6) belong to at least three distinct ancestral subgroups, suggesting an invasion of the C1 INH gene by consecutive waves of *Alu* amplification.

Studies on the C1 INH mRNA obtained from monocytes of F1 and F4 patients (32) indicate that stable transcripts from the deleted genes accumulate to normal levels and that the open reading frame of C1 INH is not altered by exon 4 deletions (14, 32). Thus one predicts that a shortened or elongated protein is synthesized in the patients described here. However, exon 4 encodes a cysteine residue (cysteine-183) that is apparently engaged in a disulfide bond (12), which may affect the stability of C1 INH or its ability to be secreted.

In our survey of 45 unrelated type I HAE kindreds, we found additional family-specific sets of altered restriction fragments, which brings to about 20% the frequency of readily detectable DNA rearrangements in type I HAE families (discussed in ref. 33). The data reported here link the occurrence of such major alterations in the C1 INH gene to the unusual abundance of intragenic *Alu* sequences.

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