Nonsense Mutations Affect C1 Inhibitor Messenger RNA Levels in Patients with Type I Hereditary Angioneurotic Edema

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Abstract

Members of two unrelated families with type I hereditary angioneurotic edema (HANE) were found to have elevated levels of C1 inhibitor (C1INH) mRNA. DNA sequence analysis of PCR-amplified monocyte C1INH mRNA revealed normal and mutant transcripts, as expected in this disorder that occurs in heterozygous individuals. Single base mutations near the 3′ end of the coding sequence were identified in affected members of each family. One mutation consisted of insertion of an adenosine at position 1304 which created a premature termination codon (TAA), whereas the second consisted of deletion of the thymidine at position 1298 which created a premature termination codon (TGA) 23 nucleotides downstream. These mutations are ~ 250 nucleotides upstream of the natural termination codon. Nuclear run-off experiments in one kindred revealed no difference in transcription rates of the C1INH gene between the patients and normals. C1INH mRNA half-life experiments were not technically feasible because of the prolonged half-life of the normal transcript. Dideoxynucleotide primer extension experiments allowed the differentiation of the normal and mutant transcripts. These studies showed that the mutant transcript was not decreased relative to the normal, and this therefore was at least partially responsible for the C1INH mRNA elevation. This elevation may be due to the decreased catabolism of the mutant transcript. (*J. Clin. Invest.* 1991. 88:755–759.) Key words: serpins • complement • premature termination codon • polymerase chain reaction • mRNA stability

Introduction

C1INH inhibitor (C1INH)[1] is a serine proteinase inhibitor that plays a central role in the regulation of both the classical complement pathway and the contact activation pathway of the intrinsic coagulation and kinin-forming systems (1). The C1INH gene has been localized to chromosome 11; it is ~ 17 kb long with a coding region of ~ 1,800 bp (2–4). It consists of eight exons interrupted by introns that contain a high density of repetitive DNA sequences (Alu repeats), thus making the C1INH gene susceptible to rearrangements (4). Inherited deficiency of C1INH is known as hereditary angioneurotic edema (HANE). The disease is transmitted as an autosomal dominant trait (5). Individuals with HANE, at the molecular level, are heterozygous for defects within the C1INH gene. The main clinical feature of the disease is localized, recurrent, acute attacks of edema of the skin and mucosa (6). Acute attacks, especially of life-threatening laryngeal edema, can be prevented with androgen therapy or reversed with C1INH concentrate (7, 8). There are two phenotypic variants of the disease. Type 1 HANE is characterized by low antigenic and functional plasma levels (~ 5–30% of the normals) of a normal C1INH protein. These patients represent the majority of HANE patients (~ 85%). Type 2 HANE is characterized by the presence of normal or elevated antigenic levels of a dysfunctional mutant protein together with reduced levels of the functional protein (9, 10). The available data suggest that a variety of mutations are responsible for C1INH deficiency. Restriction fragment length polymorphism (RFLP) of the C1INH gene has been demonstrated in a minority of patients of both types (10–15% of kindred) (11, 12). All RFLPs described in type I HANE have resulted from partial deletions or insertions within the C1INH gene and these all have involved Alu repetitive DNA (13, 14). Other mutations that may result in type I HANE have not been defined at the molecular level. Single base substitutions at the reactive center coding region have been shown to be the most common abnormality in type 2 HANE (15, 16, Aulak, K. S., personal communication), although at least two other mutations, one a single base change and the other a codon deletion, have been observed (17, 18).

Studies at the mRNA level have demonstrated that levels of C1INH mRNA are ~ 50% of normal in the majority of type I HANE patients, as might be expected with transcription of only one allele (19, 20). Some patients with deletions transcribe a small mRNA, which is present together with the normal transcript (14, 19, 21). We also have identified two kindred with elevated quantities of a normal size C1INH message, despite the low antigenic and functional plasma levels of C1INH (22). In this study, we investigated the molecular genetic defects responsible for this abnormality in these two type 1 HANE families. Sequence analysis in each revealed single base mutations leading to the introduction of premature termination codons located near the 3′ end of the gene. The abnormal transcript was increased in concentration in one family and was present in quantities similar to the normal transcript in the other. The fact that the transcription rate does not show any variation compared with the normal suggests that the abnormal message

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1. *Abbreviations used in this paper:* C1INH, C1 inhibitor; HANE, hereditary angioneurotic edema; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

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is relatively stable despite the presence of a premature stop codon.

Methods

Patients. We have investigated two affected members from each of two different type 1 HANE families. All affected members of each kindred had clinical histories of recurrent episodes of angioedema. Multiple measurements of C1INH levels in all affected individuals were always decreased, both by functional and immunochemical measurement. No RFLPs were demonstrated in these subjects (11). At the time of the analyses the patients had received no androgen or other therapy for at least one year.

RNA isolation. Total cellular RNA was isolated from adherent monocyte monolayers (23) by lysis with guanidinium isothiocyanate and centrifugation through cesium chloride density gradients (24). RNA was quantitated by absorbance at 260 nm.

Northern blot analysis. Total cellular RNA (10 μg) was denatured with formaldehyde and formamide and separated by electrophoresis in formaldehyde-containing agarose gels. Samples were transferred to nitrocellulose filters and hybridized with a C1INH cDNA probe ^{32}P-labeled by nick translation (24), or with the oligolabeling method (25) (Oligolabelling Kit; Pharmacia Fine Chemicals, Piscataway, NJ).

Sequence amplification with the polymerase chain reaction (PCR). cDNA was synthesized from 1 μg of patients' total RNA using AMV reverse transcriptase (Boehringer Mannheim, Inc., Indianapolis, IN). The cDNA was amplified using the automated PCR technique for 30 cycles with Taq I DNA polymerase (26) (Perkin-Elmer Corp., Norwalk, CT). Three sets of sense/antisense 30-bp-long oligonucleotides were synthesized with a DNA synthesizer (model 380B; Applied Biosystems, Foster City, CA). The primer sequences were designed to cover the entire C1INH cDNA coding sequence (3). Amplified cDNA was purified by electroelution (International Biotechnologies, Inc., New Haven, CT).

DNA sequencing. Amplified DNA was subcloned into pUC19 or pUC18 (Boehringer Mannheim, Inc.) and double-stranded DNA sequencing was performed by the dideoxynucleotide chain termination method (Sequenase Kit; United State Biochemical Corp., Cleveland, OH) (27).

Dideoxynucleotide primer extension of RNA. After the identification of the mutations, dideoxy primer extension was carried out essentially according to Daar and Maquat (28). With this method, primer extension is done in the presence of three deoxynucleotides and the fourth is replaced with the appropriate dideoxynucleotide. Thus, the first position at which this base is incorporated will result in termination of extension. In the experiments here, a primer complementary to nucleotides 1305–1337 was used. The experiment was carried out using 20 μg of total RNA from each patient and from a PLC/PRF/5 cell line (ATCC CRL 8024) as a control. One unit of Perfect Match Polymerase Enhancer (Stratagene, Inc., La Jolla, CA) was added to the annealing buffer before incubation. The primer was extended with ddCTP, dATP, dGTP, and dTTP (Pharmacia Fine Chemicals) by using AMV reverse transcriptase (Boehringer Mannheim, Inc.). The first cytosine incorporated by extension of this primer is at position 1293 producing a 12-nucleotide extension product. A single nucleotide deletion would be expected to result in an extension product one nucleotide shorter than the normal, whereas a single base insertion would be expected to result in an extension product one nucleotide longer. The lengths of extension products were determined by comparison with the migration of synthetic oligonucleotides run in parallel lanes.

C1INH mRNA half-life. To determine the half-life of C1INH mRNA in monocytes, gene transcription was blocked with actinomycin D (1 μg/ml) and C1INH transcripts were detected at various intervals. The cellular mortality after 16 h of treatment with actinomycin D was ~ 30–40% as assessed by trypan blue dye exclusion. Total RNA was extracted and analyzed by Northern blot (24).

Nuclear run-off. Nuclear run-off experiments were performed essentially as described (29) with some modifications. To isolate nuclei, 5.0×10^7 monocytes were washed twice with ice cold HBSS with Ca^{++} and Mg^{++} and then resuspended in 0.5 ml lysis buffer (10 mM Tris-HCl, pH 7.4, 3 mM MgCl, 10 mM NaCl, 0.5% NP-40). After 5 min incubation on ice, cells were centrifuged at 400 *g* at 4°C and resuspended in 250 μl ice-cold freezing buffer (Tris-HCl, pH 8.3, 40% glycerol, 5 mM MgCl, 0.1 mM EDTA, pH 8). 60 μl 5× run-off buffer (25 mM Tris-HCl, pH 8, 12.5 mM $MgCl_2$, 750 mM KCl, and 1.25 mM each of dGTP, dCTP, and dATP), and 100 μCi ^{32}P-UTP (6,000 Ci/mmol; Amersham Corp., Arlington Heights, IL) were added to 230 μl of nuclei suspension and incubated at 30°C for 30 min. Transcripts then were isolated using the guanidine/cesium chloride method with 50 μg yeast mRNA added as carrier. The RNA pellet was resuspended in 180 μl ice-cold TNE (0.5 M Tris-HCl, pH 8, 1.5 M NaCl) and denatured with 20 μl 2N NaOH on ice for 10 min. The solution was neutralized by the addition of Hepes pH 7.2 (final concentration, 0.48 M). RNA was ethanol precipitated and dissolved in 100 μl hybridization solution (10 mM TES, 0.2% SDS, 10 mM EDTA, 3 M NaCl). The RNA solution was hybridized at 65°C for 48 h with DNA immobilized on nitrocellulose filters. In each experiment, each filter was hybridized with the same amount of ^{32}P. The filters then were washed with 0.2× SSC at 65°C for 30 min and incubated at 37°C in 0.2× SSC with 10 Mg/ml RNase A for 30 min. Filters then were exposed for autoradiography. For immobilization of DNA to filters, 5 μg of plasmid containing C1INH cDNA was denatured with 0.3 M NaOH at 60°C for 30 min, neutralized with ammonium acetate and spotted onto nitrocellulose filters using a slot blot apparatus (Shleicher & Schuell, Keene, NH). pBR 322 DNA was used as a negative hybridization control. The plasmids used contained a murine c-*fos* genomic clone (pc-*fos*-3) (30) and a c-*jun* cDNA (clone AH119) (31) and a full-length cDNA coding for C1INH subcloned in the Hind III and Sal I sites of the Genescribe-Z pTZ18R vector.

Results

Northern blot analysis. Northern blot analysis demonstrated the presence of elevated levels of normal sized specific C1INH mRNA in the patients from the two type 1 kindred analyzed (Fig. 1). To confirm these data the analysis was repeated on samples obtained at different times from the same subjects and no variation was found (data not shown).

Sequence analysis. Sequence analysis of PCR-amplified DNA revealed the presence of single base mutations in both

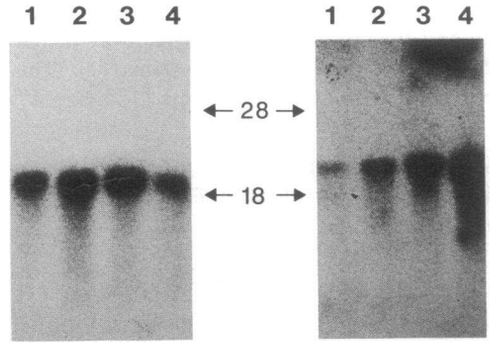

Figure 1. Northern blot analysis of actin mRNA (*left*) and C1INH mRNA (*right*) from monocyte monolayers from a normal individual (lane *2*) and three different type I HANE patients (lanes *1*, *3*, and *4*). The sample in lane *1* was from a family in which the C1INH mRNA is present at levels ~ 50% of normal (19). The samples in lanes *3* and *4* are from family 2 and family 1, respectively.

families in addition to clones with sequences corresponding to the normal allele. In the first family (Fig. 2) the mutation consisted of the insertion of an adenosine at nucleotide 1304 leading to the creation of a premature termination codon (TAA) at a position corresponding to amino acid 401 of the normal C1INH protein. In the second family (Fig. 3) deletion of a thymidine was present at position 1298 creating a premature termination codon (TGA) 23 nucleotides downstream (not shown). Interestingly, the mutations are located very near one another within the coding region in exon 8, the last exon of the C1INH gene. This region is located upstream from the C1INH reactive center (nucleotides 1331–1333), and ~ 250 nucleotides from the natural termination codon. There were no other alterations in the complete coding sequences of the mutant alleles from each of the families. In confirmation of the heterozygous nature of the defect, clones also were identified with completely normal sequences. The mutations were confirmed in both affected members of each family and on different preparations of PCR-amplified DNA.

Dideoxynucleotide primer extension. To differentiate between the transcripts from the normal and the mutated alleles and to confirm that both alleles are transcribed, we performed dideoxynucleotide primer extension (28). With this procedure, the two alleles can be distinguished on the basis of sequence heterogeneity. Using ddCTP as the terminating nucleotide the primer was extended 12 nucleotides (1304–1293) in the normal allele (Fig. 4, lane *3*), 11 nucleotides in the allele with the deletion (Fig. 4, lane *2*), and 13 nucleotides in the allele with an insertion (Fig. 4, lane *1*). This experiment allowed the demonstration that the abnormal transcript is present in concentrations equal to (family 1, Fig. 4, lane *1*) or greater than (family 2, Fig. 4, lane *2*) the concentration of the normal transcript.

Half-life and nuclear run-off. To analyze the mechanisms leading to the increased level of mRNA in these patients, we attempted to investigate both transcription rate and RNA stability. However, the RNA stability experiments were not reliable because of the long half-life (over 16 h) of the normal C1INH mRNA. The data beyond 16 h were inaccurate because of spontaneous mortality of the cells treated with actinomycin D. Nuclear run-off assays revealed no difference in the transcription rate between two different normal individuals and a member of family 1 (Fig. 5), thus indicating that the increase of total C1INH mRNA in this family was not due to an increased transcription rate. No member of family 2 was available for this study.

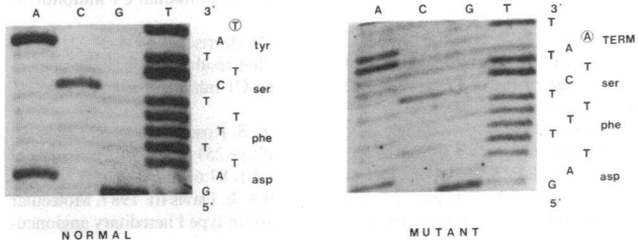

Figure 2. Sequence analysis of PCR-amplified DNA from the normal and mutant alleles of family 1. The sequence shown in each case corresponds to the coding strand. In the mutant allele, an adenosine (*circle*) is inserted between the adenosine and thymidine (*circle*) encoding tyrosine-401 of the normal allele.

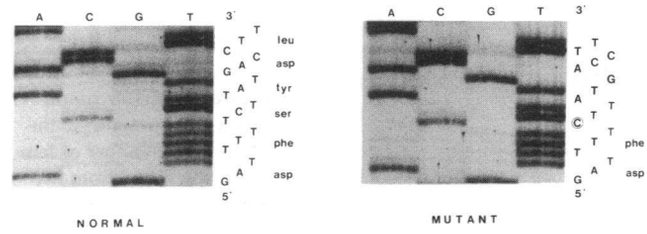

Figure 3. Sequence analysis of PCR-amplified DNA from the normal and mutant alleles of family 2. The sequence shown in each case corresponds to the coding strand. A thymidine present in the codon for serine-400 in the normal allele is deleted in the mutant allele.

Discussion

The mutations leading to C1INH deficiency in the two described families with type 1 HANE were single base substitutions that resulted in the introduction of premature termination codons in the last exon of the C1INH gene. Northern blot analysis showed elevated levels of C1INH-specific mRNA in these subjects despite the low antigenic and functional levels of C1INH protein in plasma (Fig. 1). These elevated C1INH mRNA levels were observed in multiple RNA samples from each family over a period of 3 yr (data not shown). Earlier studies have shown that the majority of kindred with type I HANE reveal levels of C1INH mRNA that are, at most, 50% of normal levels (19, 20, 32). Some patients with partial deletions reveal two transcripts: a normal mRNA present at concentrations ~ 50% of normal; and a smaller transcript of the partially deleted allele which is at similar or lower concentrations. In the two families described here, the dideoxynucleotide primer extension studies prove that both alleles are, in fact, transcribed. In one family, the normal and abnormal transcripts appear to be similar in concentration (Fig. 4, lane *1*), whereas in the

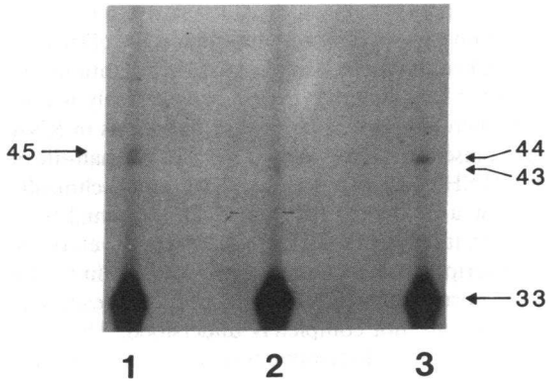

Figure 4. Dideoxy primer extension of monocyte mRNA from a normal individual (lane *3*) and from members of family 1 (lane *1*) and family 2 (lane *2*). The normal mRNA extends 11 nucleotides to guanine-1293 to give a 44-nucleotide product (lane *3*). The mRNA from family 1, the family with insertion of an adenosine between nucleotides 1303 and 1304 extends to reveal two products of approximately equal intensity, at 44 and 45 nucleotides (lane *1*). The mRNA from an affected member of family 2 reveals extension products of 44 and 43 nucleotides (lane *2*). These products were difficult to photograph, but the smaller deletion product was of much greater intensity that the normal 44 nucleotide product.

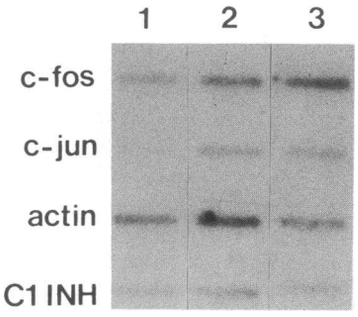

Figure 5. Nuclear runoff assays using monocyte nuclei from a member of family 1 (lane *1*) and from two different normal individuals (lanes *2* and *3*). The C1INH mRNA relative to control transcripts was not increased over the normals.

other, the mutant transcript is present in larger quantities (Fig. 4, lane *2*). Thus, the elevated mRNA levels are, at least partially, a result of an increase in concentration of the abnormal transcript. It was not possible to detect any smaller C1INH protein in sera from these patients after immunoprecipitation with anti-C1INH antiserum (data not shown). Only C1INH of normal size was observed. Therefore, it is not known whether the abnormal mRNA is not translated or if it is translated into an unstable truncated C1INH protein.

There is growing evidence that nonsense mutations are not always associated with decreased levels of steady-state mRNA, as previously reported (28, 33–35). Normal levels of mRNA have been shown for the LDL receptor (36), for the beta-globin chain (37) and for apolipoprotein CII (38) in patients with nonsense mutations of these genes. Similar findings were reported for the dihydrofolate reductase gene in which nonsense mutations were artificially introduced at various points in the gene sequence (39). These data all indicate that the closer a mutation is to the 3′ end of a gene the higher the levels of abnormal mRNA. The mechanisms leading to alterations in mRNA levels with premature termination codons are not clearly understood. They may involve differences in transcription rate, in the efficiency of processing and transport to the cytoplasm or in the stability of intracellular mRNA. In these C1INH-deficient patients, no difference in transcription rate was found compared with normal, as shown by the nuclear run-off experiments. Because no abnormality was detected in the cDNA sequence at the splice junctions, and because the mutations are located within the last exon, RNA splicing very likely is normal. There is, therefore, no evidence that alterations in RNA processing are present in these patients. Because the half-life of the normal C1INH mRNA is so long, it was not technically possible to show a prolonged half-life of the abnormal transcripts. However, it remains possible that elevated levels of the abnormal transcripts may be due to enhanced stability compared with the normal transcript. The mechanisms leading to increased stability are not completely understood. There appear to be many different determinants involved in the regulation of mRNA stability, including structures located near the 3′ end of the mRNA (such as the poly(A) tail), and modification of mRNA secondary structures (40–43). The length of the poly(A) sequence was the same in the abnormal message from the patients described here as in the normal (data not shown). Analysis of the mRNA sequences from these patients using PC/Gene (Intelligenetics, Inc., Mountain View, CA) revealed no apparent difference from the normal C1INH mRNA secondary structure. It also has been suggested that a block in protein elongation per se may be responsible for a longer half-life of some mRNA species (44). Further studies will be required to determine whether in these patients there is a relationship between translation and mRNA levels.

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