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# Identification of New Polymorphisms of the Angiotensin I–Converting Enzyme (ACE) Gene, and Study of Their Relationship to Plasma ACE Levels by Two-QTL Segregation-Linkage Analysis

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## Summary

Plasma angiotensin I–converting enzyme (ACE) levels are highly genetically determined. A previous segregation-linkage analysis suggested the existence of a functional mutation located within or close to the ACE locus, in almost complete linkage desequilibrium (LD) with the ACE insertion/deletion (I/D) polymorphism and accounting for half the ACE variance. In order to identify the functional variant at the molecular level, we compared ACE gene sequences between four subjects selected for having contrasted ACE levels and I/D genotypes. We identified 10 new polymorphisms, among which 8 were genotyped in 95 healthy nuclear families, in addition to the I/D polymorphism. These polymorphisms could be divided into two groups: five polymorphisms in the 5′ region and three in the coding sequence and the 3′ UTR. Within each group, polymorphisms were in nearly complete association, whereas polymorphisms from the two groups were in strong negative LD. After adjustment for the I/D polymorphism, all polymorphisms of the 5′ group remained significantly associated with ACE levels, which suggests the existence of two quantitative trait loci (QTL) acting additively on ACE levels. Segregation-linkage analyses including one or two ACE-linked QTLs in LD with two ACE markers were performed to test this hypothesis. The two QTLs and the two markers were assumed to be in complete LD. Results supported the existence of two ACE-linked QTLs, which would explain 38% and 49% of the ACE variance in parents and offspring, respectively. One of these QTLs might be the I/D polymorphism itself or the newly characterized 4656$(CT)_{2/3}$ polymorphism. The second QTL would have a frequency of ~.20, which is incompatible with any of the yet-identified polymorphisms. More extensive sequencing and extended analyses in larger samples and in other populations will be necessary to characterize definitely the functional variants.

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## Introduction

Angiotensin I–converting enzyme (ACE) (dipeptidyl carboxypeptidase I, DCP I; 3.4.15.1) is a zinc metalloprotease that cleaves angiotensin I into the potent vasoconstrictor hormone angiotensin II and inactivates the vasodilator peptide bradykinin (Ehlers and Riordan 1989). ACE is also able to cleave several other substrates, but the physiological relevance of this property is not known. ACE is expressed mainly as a large molecular form in endothelial, epithelial, and neuroepithelial cells (Sadoshima et al. 1993; Sibony et al. 1993). This large isoform of ACE presents two active sites located within two homologous domains, a structure resulting from an ancient duplication of the gene (Hubert et al. 1991). It is also expressed, under the control of an alternative intragenic promoter, as a smaller isoform on male germ cells (Lattion et al. 1989; Nadaud et al. 1992). This smaller isoform corresponds to the evolutionary ancestral form, the homologous gene of which was identified in *Drosophila melanogaster*, and contains only one active site (Cornell et al. 1995).

Plasma ACE levels are remarkably stable within an individual, while marked differences are observed between individuals (Alhenc-Gelas et al. 1991). A segregation analysis in healthy nuclear families of Caucasian origin revealed a major quantitative trait locus (QTL) responsible for nearly half of the variance of plasma ACE levels (Cambien et al. 1988). A polymorphic DNA marker located in the ACE gene, the insertion/deletion (I/D) polymorphism, was then shown to be strongly related to plasma ACE concentrations, the D allele being associated with raised concentrations in a codominant fashion (Rigat et al. 1990). The results of a subsequent combined segregation-linkage analysis in French families suggested that the I/D polymorphism was in strong linkage disequilibrium (LD) with an unmeasured functional mutation of the ACE gene (Tiret et al. 1992). This mutation appeared to be frequent and explained the major part of the genetic variance of plasma ACE. A similar study performed in African Caribbean families confirmed the existence of an ACE-linked QTL influencing ACE levels but also revealed a weaker LD be-

tween this QTL and the I/D polymorphism and suggested the existence of a second QTL unlinked to ACE (McKenzie et al. 1995).

The interest in identifying functional variants of the ACE gene is strengthened by the positive association found between the ACE D allele or raised ACE concentrations and several cardiovascular diseases such as myocardial infarction, left ventricular hypertrophy, or carotid-wall thickening (Cambien et al. 1992; Bonithon-Kopp et al. 1994; Schunkert et al. 1994), even though this association remains controversial (Bohn et al. 1993; Lindpaintner et al. 1995).

The aim of the present study was to identify new polymorphisms of the ACE gene that might have a functional role in determining plasma ACE levels. These polymorphisms were detected by analyzing the DNA sequences in the coding sequence and the 5′- and 3′-flanking regions of the ACE gene of selected individuals. The association of these polymorphisms with plasma ACE levels was then investigated in a sample of families that we previously analyzed for the I/D polymorphism (Tiret et al. 1992). In this earlier paper, we used combined segregation-linkage analysis to test the hypothesis that an ACE-linked QTL in LD with the I/D polymorphism explained part, or all, of the family resemblance of ACE levels. In the present paper, we extend this analysis to models specifying two ACE-linked QTLs in LD with two markers of the ACE gene.

## Subjects and Methods

### *Detection of Polymorphisms in the ACE Gene*

*Selection of Subjects.*—From our earlier study, four adult subjects were selected according to their plasma ACE levels measured on FAPGG (*F*uranacryloyl *P*he *G*ly *G*ly) substrate as described by Beneteau et al. (1986) and their ACE I/D genotypes determined by PCR (Rigat et al. 1992). Two subjects were homozygous for the D allele and had high plasma ACE levels (132 and 152 units), and two subjects were homozygous for the I allele and had low plasma ACE levels (46 and 29 units).

*Detection of Mutations.*—The map of plasmid clones containing the promoter region of the gene (pHA6.5) or its 3′ part (pHA3.2) has been published previously (Hubert et al. 1991). The sequence of the 5′ part of the ACE gene was obtained from exonuclease III–deleted plasmid pHA6.5 (Erase-a-base kit, Promega). Deleted plasmid pHA6.5 and pHA3.2 were sequenced by the dideoxy termination method (Sequenase kit version 2.0, Amersham).

Several pairs of primers were designed to amplify 5,580 nucleotides of the ACE gene upstream from the transcription start point, the coding sequence and 970 nucleotides in the 3′ part of the gene. PCR conditions and oligonucleotides used are available on request. For the 5′ region and the 3′ UTR, genomic DNA was extracted from peripheral blood leukocytes, amplified with one biotinylated and one unlabeled primer, and sequenced (Sequenase kit version 2.0, Amersham) directly after single-strand DNA preparation using streptavidin-coated magnetic particles (Dynabead, Dynal). For the coding sequence, the PCR-amplified ACE cDNA was used for sequence determination. Total RNA was extracted from peripheral blood leukocytes by use of standard techniques (Chomczynski and Sacchi 1987). First-strand cDNA was synthesized using 2 μg total RNA and MMuLV reverse transcriptase (Life Technologies) and was used for PCR amplification. PCR products were directly sequenced (Sequenase PCR sequencing kit, Amersham). Sequences differing between II and DD subjects were selected for further analysis.

### *Genotyping in Families*

Ten new polymorphisms were identified, six in the promoter region, three in the coding sequence, and one in the 3′ UTR. Eight polymorphisms were genotyped by the allele-specific oligonucleotide (ASO) hybridization method after PCR amplification. PCR primers and ASO are indicated in table 1.

All polymorphic regions, except for the A-240T and T-93C polymorphisms, were amplified by PCR in a 25-μl final volume containing 0.1 μg genomic DNA, 10 pmol of each primer, 20 mM Tris-HCl pH 8.4, 50 mM KCl, and 2 mM MgCl2. After a 5-min denaturation step, 1 U of *Taq* DNA polymerase (Life Technologies) and 50 μM dNTPs were added, followed by 30 amplification cycles (94°C, 30 s; melting temperature ($T_m$), 30 s; 72°C, 30 s) performed in a DNA thermal cycler (Techne PHC3). Final extension was performed at 72°C for 10 min. $T_m$'s for DNA amplifications were: 63°C for T-5491C and A-5466C; 61°C for T-3892C; 58°C for T1237C; 65°C for G2350A; and 66°C for 4656$(CT)_{2/3}$. The A-240T and T-93C polymorphisms were amplified by PCR in a 25-μl final volume containing 0.1 μg genomic DNA, 25 pmol of each primer, 20 mM Tris-HCl (pH 8.2), 10 mM KCl, 6 mM (NH4)2SO4, 2 mM MgCl2, 0.1% Triton X-100, and 10 μg/ml BSA. After a 5-min denaturation step, 1 U of Native *Pfu* DNA polymerase (Stratagene) and 100 μM dNTPs were added, followed by 30 amplification cycles (98°C, 1 min; 68°C, 1 min; 75°C, 3 min). PCR-amplified DNAs were denatured in 100 μl 0.4 M NaOH, 5 mM EDTA loading buffer, and spotted on Hybond $N^+$ membranes (Amersham). Membranes were hybridized overnight in 10% polyethyleneglycol (PEG), 7% SDS–containing wild-type or mutated ASO $^{32}P$ end-labeled with T4 polynucleotide kinase (New England Biolabs). Incubation temperature was specific to each ASO and was based on the empirical formula $T_m = 4(G + C) + 2(A + T) - 5$°C. Membranes were washed in 2 × SSC, SDS 0.1%

**Table 1**

**Sequences of PCR Primers and Allele-Specific Oligonucleotides (ASOs) Used for ACE Genotype Determination**

| Polymorphism | PCR Primers | PCR Product Size (bp) | ASO[a] |
|---|---|---|---|
| T-5491C | (sense) 5′CAGAGTTGTACAACCATCACT3′<br>(antisense) 5′CCGTCTTTGGAAACTTGTCTG3′ | 232 | 5′AGCCCTG(C/T)CATGCCA3′ |
| A-5466C | (sense) 5′CAGAGTTGTACAACCATCACT3′<br>(antisense) 5′CCGTCTTTGGAAACTTGTCTG3′ | 232 | 5′AGTATAT(C/A)TATGCCC3′ |
| T-3892C | (sense) 5′GAACAGGGAGGATAGCACAGG3′<br>(antisense) 5′GACCAGGCTGGCCATATGGAG3′ | 539 | 5′GGGG(T/C)TGCTGTACAT3′ |
| A-240T | (sense) 5′TGTCACTCCGGAGGCGGGAGGCT3′<br>(antisense) 5′CGGCTCTGCCCCTTCTCCTGCGC3′ | 389 | 5′CATCTTC(A/T)AAAGAGA3′ |
| T-93C | (sense) 5′TGTCACTCCGGAGGCGGGAGGCT3′<br>(antisense) 5′CGGCTCTGCCCCTTCTCCTGCGC3′ | 389 | 5′GGGGACT(T/C)TGGAGCG3′ |
| T1237C | (sense) 5′CATGAGATGGGCCATATACAG3′<br>(antisense) 5′TCAGGAGTGGAGACCGAGAGC3′ | 127 | 5′ATCTGCC(T/C)GTCTCCC3′ |
| G2350A | (sense) 5′ATCTGACGAATGTGATGGCCA3′<br>(antisense) 5′ATTGAGCCGGGCAGCCTGGT3′ | 142 | 5′TGGCCAC(G/A)TCCCGGA3′ |
| $4656(CT)_{2/3}$ | (sense) 5′GAGTACCTTGGAGGGCCTGCT3′<br>(antisense) 5′GCTACACTCCAGCGTCTGAGG3′ | 177 | $CT_2$-5′CATGACTCTGCTCG3′<br>$CT_3$-5′ATGACTCTCTGCTC3′ |

[a] Nucleotides differing between the two ASO are indicated in parentheses.

(1 × SSC = 150 mM NaCl and 15 mM sodium citrate) at $T_m$ + 5°C for 5 min and exposed for autoradiography.

### *Family Data*

A detailed description of the population study has been given by Tiret et al. (1992). The sample comprised healthy nuclear families of Caucasian origin with both parents <60 years of age and at least two offspring ≥9 years of age. Informed consent was obtained from the parents. Of the 98 initial families, 3 were excluded because of genotype incompatibility between parents and offspring for one of the newly identified polymorphisms, leaving 95 complete families ($n$ = 395). The enzymatic activity of plasma ACE was measured on a synthetic substrate (FAPGG) as described by Beneteau et al. (1986). The ACE I/D genotype was determined by PCR (Rigat et al. 1992).

### *Statistical Methods*

*Hardy-Weinberg equilibrium and linkage disequilibrium.*—Hardy-Weinberg equilibrium was tested in parents by $\chi^2$ analysis. Pairwise-linkage disequilibrium coefficients were estimated in parents by log-linear-model analysis (Tiret et al. 1991), and the extent of disequilibrium was expressed in terms of $D' = D/D_{max}$ or $D/D_{min}$ (Thompson et al. 1988).

*Analysis of variance.*—The association of each polymorphism with plasma ACE levels was tested by one-way analysis of variance, separately in parents and offspring. Analysis of variance was applied as a first approach, although offspring within families could not be considered as independent. For all polymorphisms, a codominant model assuming additive allele effects with no dominance deviation fitted well to the data. This model was adopted in subsequent analyses, and results were expressed in terms of allele effects. The association of each polymorphism with ACE levels after adjustment for the I/D polymorphism was then tested by two-way analysis of variance. For polymorphisms of the 5′ region, this approach is valid, despite their complete LD with I/D, because adjusted allele effects could be estimated within two different I/D genotypes, ID, and DD (see Family Study section). For polymorphisms of the coding region and the 3′ UTR, the results must be taken with greater caution, because of their nearly complete association with I/D. The proportion of ACE variance attributable to each polymorphism after adjustment for I/D (adjusted $R^2$) was calculated as the ratio of the sum of squares due to the polymorphism to the I/D-adjusted total sum of squares (total sum of squares minus the I/D sum of squares).

*Combined segregation-linkage analysis.*—We have elsewhere described combined segregation-linkage analysis in the case of one unmeasured functional variant in LD with one measured marker (Tiret et al. 1992). In that case, the likelihood for a family is written as

$$L(\theta/x, m) = \Sigma_g P(x, m/g)P(g) = \Sigma_g P(x/g, m)P(m/g)P(g)$$
$$= \Sigma_g P(x/g)P(m/g)P(g) .$$

In this expression, $g$ is the familial vector of genotypes at the functional locus, $m$ is the familial vector of genotypes at the marker locus, $P(g)$ is the prior probability

of the genotypes at the functional locus, $P(x/g)$ is the penetrance function conditional on the genotype, and $P(m/g)$ is function of the linkage relationship between the functional and the marker loci. A recombination rate between the two loci of 0 is assumed, since they are both situated within the ACE gene.

Let us now consider two diallelic functional loci denoted *s/S* and *r/R*. They generate four possible haplotypes *sr, sR, Sr,* and *SR*. In parents, on the assumption of Hardy-Weinberg equilibrium, $P(g)$ is a function of three independent parameters that can be expressed, for example, as $P(s)$, $P(r/s)$, and $P(r/S)$. In parents, $P(m/g)$ is function of the gametic association between the functional and the marker loci. If we consider two diallelic markers (for example, A-240T and I/D) generating four haplotypes A-I, A-D, T-I, and T-D, $P(m/g)$ can be written as a function of 12 independent parameters, which are P(A-I/*sr*), P(A-I/*sR*), ..., P(T-I/*SR*). Constraints are introduced on these parameters so that the sum of the probabilities conditional on a given functional haplotype does not exceed 1. In offspring, $P(g)$ and $P(m/g)$ are derived from the Mendelian probabilities.

Because of the nearly complete LD observed between the A-240T and I/D polymorphisms, we assumed without a great loss of information that the two markers were in *complete* LD, i.e., families in which the rare haplotype T-I was observed or deduced from family genotypes were excluded (four families). There were then only eight remaining parameters of gametic association.

Within each genotype, the penetrance function was that of the regressive model (Bonney et al. 1988). For a family, it was assumed to be multivariate normal with genotype-specific means $\mu_g$ and common matrix of variance-covariance $\Sigma$. In view of our previous findings (Tiret et al. 1992) and of the results of the present analyses of variance, we assumed a strictly codominant effect of alleles *S* and *R* on ACE levels. The genotype-specific means were then written as additive functions of *S* and *R* effects, plus a possible interaction term. Allele effects were allowed to vary between parents and offspring, since results of our earlier paper suggested that these effects were higher in offspring (Tiret et al. 1992). The diagonal elements of $\Sigma$ were the residual variances among parents and offspring and the off-diagonal elements were the covariances among relatives. These covariances could be due to any source, genetic or environmental.

*Scheme of segregation-linkage analysis.*—The first model fitted specified only one QTL, *r/R,* in LD with two markers of the ACE gene, A-240T and I/D. In this model, the frequency of *s* was fixed to 1 and its allele effects to 0. The second model specified one ACE-linked QTL, *r/R,* and a second QTL, *s/S,* unlinked to ACE. This was achieved by setting P(*r/s*) = P(*r/S*) and P(A-I/*sr*) = P(A-I/*Sr*), P(A-I/*sR*) = P(A-I/*SR*), P(A-D/*sr*) = P(A-D/*Sr*), and P(A-D/*sR*) = P(A-D/*SR*). The third model was a model assuming the cosegregation of two ACE-linked QTLs, *s/S* and *r/R,* in LD with the two markers. Because of the strong LD observed within the ACE gene, the two QTLs were assumed to be in complete LD, the *S* and *r* alleles being never associated (P[*r/S*] = 0 and P[A-I/*Sr*] = P[A-D/*Sr*] = 0). These restrictions were made because of convergence problems encountered in the unrestricted model. However, the hypothesis of complete LD between the two functional variants was later tested in a more parsimonious model. Identity between one QTL and one marker was tested by specifying appropriate constraints on the gametic association parameters. It should be noted that the particular model specifying that the two QTLs are confounded with the two markers allows one to estimate jointly the allele effects associated with the two markers while taking into account the family dependency. This is an extension of the measured genotype analysis (Boerwinkle et al. 1986) to the case of two linked markers. All hypotheses were tested by means of the likelihood-ratio criterion. We developed our own program and linked it to the GEMINI maximization procedure (Lalouel 1981).

In offspring, an adjustment of ACE levels on age and age$^2$ was made prior to analysis, separately in sons and daughters. The ACE mean in offspring was then added to the residuals. In parents, no adjustment was necessary.

## Results

### *New ACE Polymorphisms*

The 10 new polymorphisms identified by comparison of the DNA sequences of four selected subjects are shown in figure 1. All the polymorphisms were single-base transitions, except for $-4423(A)_{19/20}$, consisting of a repetition of 19 or 20 adenosine, and $4656(CT)_{2/3}$, which is a repetition of two or three CT dinucleotides. The $-4423(A)_{19/20}$ and the G2215A polymorphism, a G→A transition leading to a silent mutation in the coding sequence, were not genotyped in families, for technical reasons. The T-93C polymorphism was in complete association with A-240T and therefore was not included in the statistical analysis.

### *Family Study*

The means of age and plasma ACE level are given in table 2. ACE means were significantly higher in offspring than in parents ($P < .001$) and in sons than in daughters ($P < .001$). Age and age$^2$ explained 9% and 15% of ACE variance in sons and daughters, respectively.

None of the ACE gene polymorphisms exhibited significant deviation from Hardy-Weinberg expectations. As shown in table 3, all polymorphisms were in very

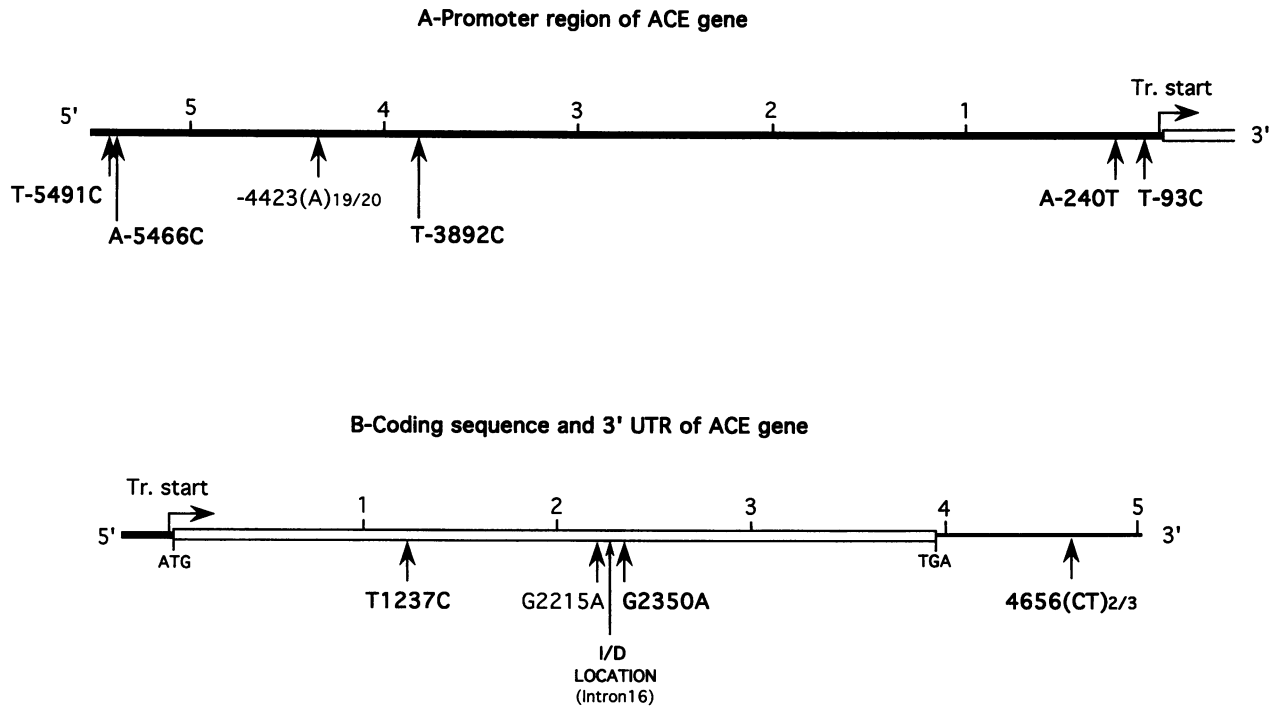


**Figure 1** Schematic representation of the ACE 5′-flanking region (*A*), coding sequence, and 3′ UTR (*B*). Arrows indicate the locations of ACE polymorphisms. Polymorphisms in bold characters are those genotyped in the families. Polymorphisms are numbered (in bp) from the transcription start site (Tr. start) for panel *A*. Nucleotides are numbered according to the cDNA sequence in panel *B*.

strong LD with each other. The eight polymorphisms defined two groups, within which gametic association was nearly complete. The first group comprised the four polymorphisms of the 5′ part of the ACE gene, T-5491C, A-5466C, T-3892C, and A-240T, which defined two main haplotypes, T-A-T-A (the most frequent) and C-C-C-T. The second group comprised the two polymorphisms of the coding sequence, T1237C and G2350A, the I/D polymorphism situated in intron 16, and the 4656(CT)$_{2/3}$ polymorphism situated in the 3′ part of the gene. Again, these four polymorphisms defined two main haplotypes, T-D-G-2rpt (the most frequent) and C-I-A-3rpt. These two groups were themselves in almost complete negative LD, the two least-common haplotypes being almost never combined. The eight polymorphisms then generated three main haplotypes, T-A-T-A-C-I-A-3rpt, T-A-T-A-T-D-G-2rpt, and C-C-C-T-T-D-G-2rpt, whose frequencies were .343, .101, and .345, respectively. The eight polymorphisms yielded a total heterozygosity of .74.

By one-way analysis of variance, each polymorphism was significantly associated with ACE levels in a codominant fashion, and estimates of allele effects are given in table 4. In absolute values, the mean allele effects appeared higher in offspring than in parents, although the proportion of variance explained by each polymorphism ($R^2$) was of similar magnitude. By one-way analysis of variance, the I/D polymorphism was associated with the highest $R^2$ (31% and 29% in parents and offspring, respectively), but the 4656(CT)$_{2/3}$ polymorphism yielded very close results. After adjustment for the I/D polymorphism, the four polymorphisms of the 5′ region remained significantly associated with ACE levels, although allele effects and $R^2$ dramatically decreased. The interaction between each of these polymorphisms and the I/D polymorphism was never significant, indicating that the effects of polymorphisms on ACE levels were fairly additive. As an example, the mean plasma ACE levels in the combined genotypes at I/D and A-240T loci are shown in table 5. The two polymorphisms of the coding sequence were no longer associated with ACE levels after adjustment on I/D, while 4656(CT)$_{2/3}$ displayed a significant effect only in parents. However, as

## Table 2

**Mean (SD) Values of Age and Plasma ACE Level**

| | Fathers ($n$ = 95) | Mothers ($n$ = 95) | Sons ($n$ = 120) | Daughters ($n$ = 85) |
|---|---|---|---|---|
| Age (years) | 41.4 (4.2) | 39.4 (3.5) | 14.2 (3.0) | 14.4 (3.2) |
| Plasma ACE (IU/liter) | 89.5 (29.5) | 84.5 (27.5) | 126.8 (45.5) | 106.7 (39.0) |

## Table 3

**Allele Frequencies and Pairwise Linkage Disequilibrium Coefficients Between ACE Gene Polymorphisms, Estimated in Parents**

| Polymorphism | Allele Frequencies | +/− D′ | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | T-5491C | A-5466C | T-3892C | A-240T | T1237C | D/I | G2350A |
| T-5491C | .62/.38 | ... | ... | ... | ... | ... | ... | ... |
| A-5466C | .62/.38 | 1.00 | ... | ... | ... | ... | ... | ... |
| T-3892C | .56/.44 | .97 | .97 | ... | ... | ... | ... | ... |
| A-240T | .61/.39 | .99 | .99 | .96 | ... | ... | ... | ... |
| T1237C | .59/.41 | −.95 | −.95 | −.96 | −.93 | ... | ... | ... |
| D/I | .57/.43 | −.93 | −.94 | −.90 | −.92 | .88 | ... | ... |
| G2350A | .58/.42 | −.98 | −.98 | −.89 | −.96 | .85 | .94 | ... |
| $4656(CT)_{2/3}$ | .57/.43 | −.82 | −.83 | −.79 | −.81 | .78 | .91 | .86 |

NOTE.—All coefficients are significantly different from 0 at $P < 10^{-3}$; total heterozygosity = .74.

already outlined in Subjects and Methods, this last result should be interpreted with caution, because of the colinearity of the two variables due to the nearly complete association between the I/D and the $4656(CT)_{2/3}$ polymorphisms.

These first analyses suggested that none of the newly identified polymorphisms could be the functional variant inferred from our previous combined segregation-linkage analysis, since none of them explained a larger part of the variability of ACE levels than did I/D. Moreover, the additive effects on ACE levels of the I/D polymorphism and each of the polymorphisms of the 5′ region (let us consider A-240T, for example) were compatible with two different hypotheses:

1. The existence of a single unmeasured functional mutation, *r*/*R*, in complete LD with the two polymorphisms, the *R* allele always carrying the D allele and being subdivided by A-240T into two haplotypes *S*-A-D and *R*-T-D, and the *r* allele always carrying the

## Table 4

**Association between Plasma ACE Level and ACE Gene Polymorphisms**

| Polymorphism[a] | | | Nonadjusted[b] | | | Adjusted for I/D[c] | | |
|---|---|---|---|---|---|---|---|---|
| | | | Allele Effect (SE) | $R^2$ (%) | $P$ | Allele Effect (SE) | $R^2$ (%) | $P$ |
| T-5491C | (C) | Parents | 17.7 (3.0) | 16.0 | $<10^{-4}$ | 6.0 (3.2) | 1.8 | .07 |
| | | Offspring | 25.2 (3.7) | 18.3 | $<10^{-4}$ | 9.2 (4.4) | 2.1 | .04 |
| A-5466C | (C) | Parents | 18.7 (3.0) | 17.8 | $<10^{-4}$ | 6.8 (3.3) | 2.2 | .04 |
| | | Offspring | 26.1 (3.7) | 19.9 | $<10^{-4}$ | 10.5 (4.4) | 2.7 | .02 |
| T-3892C | (C) | Parents | 20.9 (2.8) | 24.3 | $<10^{-4}$ | 7.9 (3.4) | 2.9 | .02 |
| | | Offspring | 28.3 (3.6) | 23.5 | $<10^{-4}$ | 12.3 (5.1) | 2.9 | .02 |
| A-240T | (T) | Parents | 19.0 (2.9) | 18.9 | $<10^{-4}$ | 7.5 (3.2) | 2.8 | .02 |
| | | Offspring | 27.4 (3.6) | 21.8 | $<10^{-4}$ | 12.7 (4.4) | 3.9 | .005 |
| T1237C | (T) | Parents | 19.5 (2.8) | 20.4 | $<10^{-4}$ | −1.8 (4.7) | .1 | .70 |
| | | Offspring | 25.2 (3.4) | 21.5 | $<10^{-4}$ | 5.6 (5.2) | .6 | .28 |
| D/I | (D) | Parents | 23.3 (2.5) | 31.0 | $<10^{-4}$ | ... | ... | ... |
| | | Offspring | 29.5 (3.2) | 28.8 | $<10^{-4}$ | ... | ... | ... |
| G2350A | (G) | Parents | 20.9 (2.6) | 25.8 | $<10^{-4}$ | −.6 (6.5) | .0 | .93 |
| | | Offspring | 26.6 (3.5) | 22.3 | $<10^{-4}$ | 2.8 (6.6) | .1 | .67 |
| $4656(CT)_{2/3}$ | (2) | Parents | 22.5 (2.5) | 30.0 | $<10^{-4}$ | 11.7 (5.7) | 2.3 | .04 |
| | | Offspring | 27.6 (3.4) | 24.6 | $<10^{-4}$ | 7.2 (7.0) | .5 | .31 |

[a] For each polymorphism, the allele associated with raised ACE levels is given in parentheses.
[b] One-way analysis of variance assuming additive allele effects for each polymorphism.
[c] Two-way analysis of variance (for each polymorphism, allele effect, and $R^2$ are adjusted for the I/D polymorphism effect).

**Table 5**

**Mean Plasma ACE Levels according to the Combined Genotypes at I/D and A-240T Loci**

| Group and A-240T Genotype | Mean Plasma ACE Level I/D Genotype[a] (IU/liter) | | | |
|---|---|---|---|---|
| | II | ID | DD | All |
| Parents: | | | | |
| AA | 62.7 ($n$ = 28) | 75.6 ($n$ = 28) | 102.6 ($n$ = 9) | 73.8 ($n$ = 65) |
| AT | 51.0 ($n$ = 4) | 86.5 ($n$ = 70) | 102.0 ($n$ = 27) | 89.2 ($n$ = 101) |
| TT | ... | ... | 114.7 ($n$ = 23) | 114.7 ($n$ = 23) |
| All | 61.2 ($n$ = 32) | 83.4 ($n$ = 98) | 107.0 ($n$ = 59) | 87.0 ($n$ = 189) |
| Offspring: | | | | |
| AA | 80.4 ($n$ = 40) | 110.1 ($n$ = 32) | 122.3 ($n$ = 10) | 97.1 ($n$ = 82) |
| AT | 147.7 ($n$ = 1) | 122.2 ($n$ = 58) | 142.2 ($n$ = 36) | 130.0 ($n$ = 95) |
| TT | ... | 113.8 ($n$ = 2) | 150.6 ($n$ = 26) | 148.0 ($n$ = 28) |
| All | 82.0 ($n$ = 41) | 117.8 ($n$ = 92) | 142.5 ($n$ = 72) | 119.3 ($n$ = 205) |

[a] $n$, in parentheses, refers to number of subjects.

A allele and being subdivided by I/D into two other haplotypes, $r$-A-I and $r$-A-D. This situation would be compatible with the nearly complete LD between I/D and A-240T, the I and T alleles being almost never associated.

2. The existence of two different functional mutations, $s/S$ and $r/R$, within the ACE gene, one of these mutations being in strong LD with I/D and the other one with A-240T.

To elucidate which of these two hypotheses was better supported by the data, we performed a series of combined segregation-linkage analyses based on a model specifying, in its most general form, two unmeasured functional variants of the ACE gene and two measured markers in LD with these mutations. The two above hypotheses corresponded to submodels nested in this general model. As stated in Subjects and Methods, the two variants were assumed to be in complete LD. They were also considered to act additively on the phenotype, i.e., without interaction. This hypothesis was necessary because of the complete disequilibrium between the two variants which did not make possible to test an interaction. Residual family correlations were fixed to 0 to start analysis.

Analyses were first performed using the I/D and the A-240T polymorphisms as markers, since the A-240T polymorphism was associated with the highest adjusted $R^2$ in the two-way analyses of variance controlling for the I/D polymorphism. Two families were excluded because of missing combined genotype information in one or several subjects. Four additional families were deleted because of the assumption of complete LD between the two markers (see Subjects and Methods), leaving 89 families for the analysis.

The results of the segregation-linkage analyses are shown in table 6. The first model fitted specified the segregation of one single QTL in LD with the two markers. In model 2, a second QTL that was unlinked to the ACE locus was modeled. Although this model did not correspond to any of the two hypotheses stated above, it was fitted to test the hypothesis proposed by McKenzie et al. (1995) of two independent QTLs controlling ACE levels. In our data, this hypothesis was not significantly better supported than the hypothesis of one QTL ($\chi^2$ = 6.48, 3 df). Model 3 specified the cosegregation of two ACE-linked QTLs in LD with the two markers. In this model, the two parameters corresponding to the frequencies of the marker haplotypes A-I and A-D conditional on the functional haplotype $SR$ converged to 0, which suggests that the remaining haplotype T-D was always associated with $SR$. Compared to this model, the model assuming only one QTL (model 1) was strongly rejected ($\chi^2$ = 18.24, 3 df; $P < .001$). From model 3, allele frequencies at the two putative functional loci were estimated as .83/.17 for $s/S$ and .49/.51 for $r/R$, respectively.

In light of the allele frequencies estimated for $r/R$, we hypothesized that this locus might be identical to I/D. This hypothesis was not rejected (model 4 vs. model 3: $\chi^2$ = 7.76, 3 df; NS). On the other hand, the hypothesis that $r/R$ might be identical to A-240T was strongly rejected ($\chi^2$ = 51.04, 3 df; $P < .001$; model not shown). Finally, model 5 assuming that $s/S$ was identical to A-240T and $r/R$ was identical to I/D was also rejected ($\chi^2$ = 19.08, 4 df; $P < .001$).

In model 4 assuming I/D = $r/R$, which was the most parsimonious model, we relaxed the constraint of complete LD between the two functional variants, and the probability P($r/S$) was not significantly different from 0 ($\chi^2$ = 0.60, 1 df). In model 4, the allele $R$ effects were not different between parents and offspring ($\chi^2$ = 0, 1 df), while the allele $S$ effects were significantly higher in

## Table 6

**Combined Segregation-Linkage-Analysis Testing Transmission of Two ACE-Linked QTLs in Linkage Disequilibrium with I/D and A-240T Polymorphisms**

| | Model | | | | |
|---|---|---|---|---|---|
| Parameter | 1 | 2 | 3 | 4 | 5 |
| Frequency of functional allele *s* | [1] | .973 | .826 | .805 | .615 |
| Frequency of functional allele *r*: | | | | | |
| Conditional on allele *s* | .554 | .588 | .597 | .520 | .680 |
| Conditional on allele *S* | [0] | (.588) | [0] | [0] | [0] |
| Frequency of marker haplotype A-I: | | | | | |
| Conditional on haplotype *sr* | .717 | .711 | .751 | [1] | [1] |
| Conditional on haplotype *sR* | .047 | 0[a] | .144 | [0] | [0] |
| Conditional on haplotype *Sr* | [0] | (.711) | [0] | [0] | [0] |
| Conditional on haplotype *SR* | [0] | (0)[a] | 0[a] | [0] | [0] |
| Frequency of marker haplotype A-D: | | | | | |
| Conditional on haplotype *sr* | .158 | .159 | .122 | [0] | [0] |
| Conditional on haplotype *sR* | .244 | .250 | .410 | .509 | [1] |
| Conditional on haplotype *Sr* | [0] | (.159) | [0] | [0] | [0] |
| Conditional on haplotype *SR* | [0] | (.250) | 0[a] | [0] | [0] |
| Allele *S* effect: | | | | | |
| Parents | [0] | 49.13 | 8.20 | 15.89 | 8.98 |
| Offspring | [0] | 18.92 | 38.36 | 38.01 | 16.55 |
| Allel *R* effect: | | | | | |
| Parents | 32.78 | 31.55 | 32.01 | 17.76 | 17.20 |
| Offspring | 41.56 | 43.18 | 25.87 | 18.30 | 19.34 |
| Residual SD: | | | | | |
| Parents | 17.33 | 16.04 | 14.91 | 22.09 | 23.08 |
| Offspring | 30.06 | 29.40 | 26.96 | 29.78 | 34.35 |
| Alternate model | ... | 1 | 1 | 3 | 3 |
| $\chi^2$ | ... | 6.48 | 18.24 | 7.76 | 19.08 |
| df | ... | 3 | 3 | 3 | 4 |
| *P* | ... | NS | $<10^{-3}$ | NS | $<10^{-3}$ |

NOTE.—Square brackets indicate that parameter is fixed to value shown; parentheses indicate that parameter is constrained; NS = not significant; model 1 = one ACE-linked QTL; model 2 = one ACE-linked QTL (*r*/*R*) and one unlinked QTL (*s*/*S*); model 3 = two ACE-linked QTLs; model 4 = model 3 with the constraint I/D = *r*/*R*; model 5 = model 3 with the constraints A-240T = *s*/*S* and I/D = *r*/*R* (this model also allows one to test the effects associated with the two markers).

[a] Parameter estimated to a boundary value.

offspring than in parents ($\chi^2$ = 5.40, 1 df; $P < .05$). Last, including residual family correlations did not significantly improve the likelihood ($\chi^2$ = 1.28, 4 df; model not shown).

The most-parsimonious model (model 4) supported the existence of two ACE-linked QTLs controlling ACE levels, one being in complete association with the I/D polymorphism and the other one having allele frequencies ~.80/.20, incompatible with any of the yet-identified polymorphisms of the ACE gene. Together, these two variants would explain 38% and 49% of the variability of ACE levels in parents and offsprings, respectively, compared to 32% explained by the combination of I/D and A-240T.

Because the variant *r*/*R* might be a polymorphism in complete association with I/D, we repeated the same analysis by successively replacing I/D with the three other polymorphisms of the second group of polymorphisms. In analyses combining A-240T with T1237C, the identity between *r*/*R* and T1237C was rejected ($\chi^2$ = 11.62, 3 df; $P < .01$). The same conclusion was drawn from analyses combining A-240T with G2350A ($\chi^2$ = 12.14, 3 df; $P < .01$). By contrast, the hypothesis of complete association between *r*/*R* and 4656$(CT)_{2/3}$ was not rejected ($\chi^2$ = 5.66, 3 df; NS). The locus *r*/*R* might be thus either I/D or 4656$(CT)_{2/3}$, or a third locus in tight association with them.

Because results from the two-way analysis of variance were very close for the T-3892C and the A-240T polymorphisms and the highest $R^2$ for A-240T was based on children's levels, which are not independent, we repeated the analysis by combining the T-3892C and the I/D polymorphisms. Conclusions similar to those obtained with A-240T were drawn.

## Discussion

Our previous results, which showed that an ACE-linked QTL was responsible for the major part of the genetic variance of plasma ACE, justified an extensive search for the functional variant inside the ACE gene (Tiret et al. 1992). Our strategy was based on DNA sequencing of selected individuals, because this approach offers the advantage of a 100% sensitivity. Two DD and two II individuals having highly contrasted ACE levels were chosen, since the I/D polymorphism has been shown in our previous study to have very high sensitivity and specificity (Tiret et al. 1992). This selection increased the probability of detecting sequence polymorphisms in LD with both the putative functional variant and the I/D polymorphism. Because only four individuals were sequenced, some rare polymorphisms of the ACE gene could have been missed. However, we were a priori looking for common mutations.

The regions on which we focused our search were chosen according to two kinds of results. First, ACE concentrations are modulated by the ACE I/D polymorphism in both plasma and cells, which suggests that the molecular mechanisms of secretion process are not involved. Second, our previous studies showed that the ACE I/D polymorphism was associated with plasma ACE activity and with the number of immunoreactive ACE molecules (Rigat et al. 1990). These two observations instead supported the hypothesis of a polymorphism acting quantitatively at an early stage of gene expression, i.e., ACE transcription or mRNA stability. However, the coding region was also completely investigated because the possibility of a functional mutation in the coding sequence could not be ruled out, since $K_m$ or $K_{cat}$ values (the latter being influenced by enzyme concentration) had not been determined on pure plasma enzyme from subjects carrying the I or the D allele.

The 5′ flanking region was explored up to ~5.5 kb, since we had previously shown, by transfection assays, that this region contained functional elements of regulation in the first 1.2 kb 5′ to the transcription start (Hubert et al. 1991). However, even with the limitation of a cellular model, it was likely that additional *cis*-regulatory elements existed, since this region induced a rather low level of expression in cultured cells transfected with the ACE promoter fused to a reporter gene (Testut et al. 1993).

Two groups of polymorphisms were identified, a first group located in the 5′ region (from T-93C to T-5491C) and a second group in the coding sequence and the 3′ region (from T1237C to 4656[CT]$_{2/3}$). Apart from their location in the gene, the distinction between these two groups was based on two kinds of observations. First, within each group, the polymorphisms were in nearly complete association, while the two groups were in strong negative LD one with each other. Second, the 5′ polymorphisms remained significantly associated with ACE levels after adjustment for the I/D genotype, whereas polymorphisms of the second group had no longer effect after adjustment. These observations were compatible either with the presence of a single ACE-linked QTL in complete LD with each group of markers or with the presence of two ACE-linked QTLs acting additively on the phenotype.

These different hypotheses were investigated by combined segregation-linkage analysis extended to models specifying two ACE-linked QTLs in LD with two markers, each one selected in a different group of markers. In order to reduce the complexity of models, we assumed complete LD between the two markers, on one hand, and between the two functional QTLs, on the other hand. The first assumption probably had no significant impact on the results, in light of the nearly complete LD between markers and the very small number of subjects excluded from analysis. The second assumption of complete LD between the two QTLs was justified by the extremely strong LD between polymorphisms observed within the ACE gene in our population. Although this hypothesis did not seem too unrealistic, it is difficult to assess precisely the possible biases that it might have introduced. We can expect that the conclusion for a two-QTL model would still hold, even though this hypothesis was relaxed, since the $\chi^2$ resulting from the comparison of the two- and the single-QTL models would be $\geq 18.24$ with 6 df ($P = .006$). But it is more difficult to anticipate the impact on the frequencies of the two functional mutations, and, for this reason, the interpretation that one of the putative variants could be the I/D or the 4656(CT)$_{2/3}$ polymorphism must be taken with caution. A larger sample of families would be necessary to be able to estimate and to test the extent of LD between the two variants.

Evidence for two QTLs controlling plasma ACE concentrations was previously reported in a study of Jamaican families in which only the I/D polymorphism was assessed (McKenzie et al. 1995). However, in that study, the second QTL was a priori assumed to be unlinked to the ACE gene. It would be interesting to see whether the conclusions of the Jamaican study would be modified when considering a possible linkage to the ACE locus. The existence of a second QTL unlinked to ACE was not supported in French families. Even if other QTLs are involved in the control of plasma ACE levels, their effects in this population should be rather weak, as attested by the residual family correlations virtually equal to 0.

It has been shown by simulations that single-gene models may plausibly fit data generated under two-locus models (Dizier et al. 1993). This probably explains why our previous studies supported evidence for one QTL

(Cambien et al. 1988, 1994; Tiret et al. 1992). The resolution of parameters under a two-locus model was greatly improved in the present study because information from two linked markers was included in the analysis. Use of multiple tightly linked markers in population or family-based studies of genetic quantitative traits is generally advocated because the combination of several markers may help to characterize a specific haplotype likely to carry a functional mutation. However, in the present study, the two markers, rather than defining an interesting haplotype, provided information about the two QTLs independently from each other. This was possible because the two markers selected for analysis were in strong LD but not in complete association, and therefore allele effects associated with one polymorphism could be estimated while controlling for the other. Taking into account known sources of genetic variability has been shown to increase the power of detecting other genes involved in the determination of multifactorial traits (Thein et al. 1994; McKenzie et al. 1995).

The results from segregation-linkage analysis raised the possibility that the I/D or the 4656$(CT)_{2/3}$ polymorphism might be one of the putative QTLs, and in vitro studies will be required to demonstrate the functionality of these polymorphisms. The presence or absence of the 287-bp *alu*-repetitive sequence inside intron 16 of the ACE gene, which corresponds to the I/D polymorphism, could modify the stability or the splicing efficiency of the ACE mRNA precursor. On the other hand, we did not observe any effect of the I/D polymorphism on the ACE concentration measured in male germinal cells, although the germinal ACE mRNA is transcribed from an alternative promoter located in intron 12 and contains the subsequent exons of the gene, including intron 16. The 4656$(CT)_{2/3}$ polymorphism is located in the part of the 3′ UTR, which was shown to be absent in the rabbit germinal ACE mRNA, but does not correspond to any known sequence that could modify the stability of the messenger RNA.

Our results imply that other functional polymorphisms should be found in the ACE gene, since at least one of the two putative QTLs was not identified. We cannot exclude the possibility that the putative functional variant *s/S* is located in the regions already explored, since, from model 4, it can be inferred that the *S* allele would be present in only one-third of the D alleles. The probability of missing it in sequencing four D alleles would have been .20, if the sequenced alleles had been randomly chosen. In reality, this probability is probably lower because D alleles were selected on the basis of high ACE levels; therefore, increasing the probability of carrying the functional mutation. Nevertheless, the regions already explored will have to be reanalyzed in individuals selected according to the results of the present analysis. The conventional strategy that we used needs also to be extended and guided, for example, by the identification of DNAse-hypersensitive sites in order to find regulatory regions that could be located faraway from the transcription start, in both the 5′- and the 3′-flanking regions.

It is interesting to note that the plasma ACE concentration is under tight genetic control not only in humans but also in the rat, as demonstrated by the contrasted levels observed between different rat strains (Kreutz et al. 1995). Taken together with our results, this indicates that the ACE gene has in different species a tendency to present functional polymorphisms, which could result from genetic drift or positive selection pressure. ACE is indeed a key enzyme of the renin-angiotensin and kallikrein systems. The effect of an increased ACE level on vasoconstriction and cell proliferation, which may have deleterious consequences in pathological conditions, might be (or have been) beneficial in other contexts, as for example at particular stages of development (ACE increased during adolescence) or during some periods of human evolution, in particular by favoring wound healing.

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