Biochemical and Biophysical Research Communications **266,** 110–114 (1999)
Article ID bbrc.1999.1769, available online at http://www.idealibrary.com on IDEAL®

Genomic Organization of the Human γ Adducin Gene

Lorena Citterio,* Tiziana Azzani,* Stefano Duga,† and Giuseppe Bianchi*
**Division of Nephrology, Dialysis and Hypertension, San Raffaele Hospital, Milan, Italy; and*
†Veterinary Physiology and Biochemistry Institute, University of Milan, Milan, Italy

Received October 16, 1999

We report the genomic structure of the human γ adducin gene (ADD3). Adducin is a protein involved in cytoskeletal assembly and composed of α–β or α–γ subunits which share a high degree of homology between human and rat. Mutations in α subunit have been shown associated to both human and rat hypertension. The human ADD3 gene spans over 20 kb and is composed of at least 13 introns and 14 exons covering the entire coding region. The exon size ranges from 81 bp to greater than 293 bp and the intron size from 111 bp to longer than 3.2 kb. We also demonstrate the presence of an alternative splicing event around exon 13, whose sequence, position, and expression is analogous in rat Add3 gene. Moreover, human ADD3 amino acid sequence presents 91.9% of identity compared to rat sequence. Characterization of human ADD3 gene provides an important tool for mutation analysis.

Adducin is an heterodimeric cytoskeleton protein involved in the formation of actin-spectrin lactice, actin polimerization and signal transduction (1, 2). The protein is composed of related but not identical subunits (α, β and γ); moreover α and β subunits show a very high homology between humans and rats (91.3% and 91.7% respectively).

It has been postulated that adducin may partecipate in the regulation of blood pressure. In hypertensive rats of the Milan strain (MHS) a primary increase of renal tubular Na^+ reabsorption (3) is involved in the development of hypertension. In particular, the MHS strain shows an increased activity and expression of Na,K-pump units per cell compared to the normotensive MNS control (4). A missense mutation in α subunit (F316Y) is genetically associated to hypertension and this effect is modulated by mutations in β subunit (Q529R) (5) and in γ subunit (Q572K) (L. Zagato personal communication). Transfection studies have demonstrated that the α–β hypertensive variant of the protein modulates actin assembly and increases the surface expression and the activity of the Na,K-pump (6).

In humans the α-adducin locus is associated with and linked to essential hypertension (7–9). A missense mutation in this subunit (G460W) is associated with hypertension and affects the relationship between renal Na^+ excretion and blood pressure (10). Recent results indicate a direct and specific interaction between adducin and Na,K-ATPase in vitro; both rat and human adducin polymorphisms differently modulate renal Na,K-ATPase in vitro (11).

α, β and γ-adducin genes (ADD1, ADD2, ADD3) are localized on different chromosomes in both humans and rats (12–14) and are differently expressed in a variety of tissues. α-adducin is expressed in all tissues examined and may coexist as α–β and/or α–γ heterodimers depending on the expression levels of β and γ genes in the different tissues (15). A transcript of 4 kb has been detected in human kidney mRNA using an adducin-like human cDNA clone as probe (16), confirming the expression signal of α subunit and the absence of β signal in renal tissue (12).

The expression of human ADD3 in the kidney suggests that ADD3 could have a role also in human hypertension. Therefore we set out to establish the exon-intron organization of the human ADD3 ORF for genomic DNA based mutation detection.

MATERIALS AND METHODS

Identification of human ADD3 and chromosomal mapping using database analysis. Human gamma adducin cDNA (complete coding sequence Accession Number U37122), obtained by database searches using locally maintained Genbank/EMBL database, was used to perform a BLASTN search against the STSs in the nonredundant Database of Genbank STS Division. Only one STS (Accession Number G03246) showed a strong similarity to ADD3 cDNA. Further information were also reported: the linked markers and distance expressed in centiRadios from the top of chromosome linkage group, YAC clones issued by CEPH library.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the Accession Numbers Y14372 through Y14384.

0006-291X/99 $30.00

Genomic organization and DNA sequencing. (1) The YAC clones 741E1, 803A2 and 801G4 were found to contain the STS G03246 and consequently the genomic sequence of ADD3.

Almost all of the genomic region corresponding to ADD3, except part of 5′ and 3′ untraslated region, exon 1 and intron 1, was amplified using polymerase chain reaction (PCR), human genomic DNA or the YAC DNAs as templates and 10 PCR primer sets. The PCR primers were designed on the basis of the cDNA sequence and the high similarity of location of the introns between human alpha and beta adducin predicted amino acid sequences (17). The size of the introns was defined by PCR using adjacent exonic primers.

Exon 1, exon/intron junctions of intron 1 and of intron upstream exon 1 were instead defined by single-specific-primer polymerase chain reaction (SSP-PCR) (18). SSP-PCR was performed using a vector-specific generic primer common for all amplifications and a primer specific for the ADD3 cDNA sequence different for each amplification. The template of these reactions was an aliquot of ligation mixes of human genomic DNA or YAC DNA and pBluescript SK(−) digested with different restriction enzymes.

(2) All PCR reactions were performed with Taq DNA Polymerase (Bioline UK) in buffer ATG* (67 mM Tris-HCl, pH 8.8, 16 mM $(NH_4)_2SO_4$, 0.05% NP-40, 0.01% Tween 20, 5 mM β-mercaptoethanol) for 30 cycles at 96°C (1 min), annealing temperature according to the melting temperature of the appropriate set of primers, and 72°C (5 min). The PCR was carried out with a Gene Amp PCRsystem 9600 (Perkin Elmer, Norwalk, CT).

(3) We defined the exon/intron junctions by direct sequencing of PCR products. Fragments amplified were sequenced from both ends. Sequence reactions were performed either manually by use of Sequenase 2.0 DNA Sequencing Kit (USB) and (^{33}P)dATP for labelling or by use of DyeDeoxy terminator cycle sequencing and analyzed on an ABI 373A automated sequencer (Applied Biosystem, Inc) according to the procedure recommended by the manufacturer.

In order to confirm the gene structure each exon has been then amplified individually using intronic primers and checked in size and sequence. Nucleotide sequence alignment was performed using USC Sequence Alignment Server (http://www-hto.usc.edu/software/seqaln/seqaln-query.html) while aminoacidic alignment was carried out using the program SIM (http://www.expasy.ch/tods/sim-prot.html).

RT-PCR. Total RNA was prepared according to standard protocols (19). cDNA was synthesized from 2 μg of total RNA isolated from different human tissues (kidney, heart, brain, lung and blood) using the First Strand cDNA synthesis kit (Pharmacia Biotech). First-strand cDNA was used as template in a 40 μl PCR containing 1 mM $MgCl_2$, 100 ng each primer, 200 μM dNTPs, ATG* buffer and 1 unit of Taq DNA polymerase (Bioline UK). PCR conditions were 28 cycles of 30 s at 94°C, 20 s at 58°C, and 30 s at 72°C, after an initial denaturation of 4 min at 94°C. The sense primer sequence was TAGCAGGTGCAGGTGGAGTA (gamma 3—nucleotides 1103–1122) and the antisense primer sequence was CCCCCAATCTGATCTACCAT (gamma 4—nucleotides 2262–2243). The following oligonucleotide primers were instead used to amplify the alternative spliced region (exon 12–14): gamma 15, CGAACGTAAACAACAAGGCCT (nucleotides 1842–1862) and gamma 10, TTGCCATTTACTACCATGAC (nucleotides 1934–1915).

The reaction products were analysed on a 2% agarose gel stained with ethidium and specific bands were cut and extracted with Qiaex (QIAGEN In. CA) and directly sequenced.

RESULTS AND DISCUSSION

By examining the GenBank and EMBL databases we found that U37122 represents the complete coding sequence of human ADD3. The sequence lacks of complete 5′ and 3′ UTR and shows a 2025-bp open reading frame corresponding to a predicted protein product that spans 675 amino acids. BLASTN analysis of the human nonredundant Database of Genbank STS Division have revealed that a 99-nt central position of ADD3 cDNA sequence (an hypothetical exon) was identical to G03246 STS.

The STS content mapping information allowed us to identify some YACs containing ADD3 region. 741E1, 801G4 and 803A2 YAC clones derived from CEPH library were assessed to contain ADD3 genomic region by PCR. This method is a rapid way to identify the YACs containing a given marker or region among all the YACs and to obtain a good template for characterization at the genomic human level.

The human ADD3 gene spans over than 20 kb of DNA and is composed of at least 13 introns and 14 exons covering the entire coding region. We used PCR products amplified from human genomic or YAC DNA to reveal the exon-intron boundaries. The sequence of all spliced sites shown in Table 1, conforms to the GT/AG rule (20). The exon size ranges from 81 bp (exon 4) to greater than 293 bp (exon 14). The G03246 STS previously described contains ADD3 exon 7 and partial flanking intron sequences.

The introns range from 111 bp in size to larger than 3.2 Kb. Their length, except for intron 1, was determined by PCR amplification using primer sets specific to consecutive exons. Intron 6 is the smallest and was completely sequenced, whereas the central parts of other introns were not sequenced. The introns closer to the 5′ end of the gene tend to be larger.

The nucleotide sequences we have produced have been deposited in GenBank (Accession Nos. Y14372 through Y14384).

Comparison between U37122 coding sequence and our DNA sequences revealed a few differences. We have found three nucleotide substitutions (nucleotide position 1217, 1390 and 1585) and three nucleotide deletions (nucleotide position 1408, 1423 and 1433) with lost of one amino acid. However the resulting ORF spans 2022 bp corresponding to 674 amino acids. By RT-PCR from peripheral blood leukocyte RNA we demonstrated the presence of a further 96 bp sequence compared to both U37122 and D67031 coding sequences. This in-frame insertion corresponds to exon 13 and encodes 32 amino acids between codons 578 and 579. The resulting ORF becomes 2118 bp corresponding to 706 amino acids.

Since this alternative splicing event around exon 13 creates two different transcripts, primers corresponding to sequences of exons 12 and 14 were used in an RT-PCR in order to generate two distinct bands: the upper band (ADD3a) contained exons 12, 13 and 14, while the lower band (ADD3b) contained only exons 12 and 14. The same reaction performed on human kid-

TABLE 1

Splice Junction Sequences, Exon Sizes, and Estimated Intron Sizes of ADD 3

Exon	3′ splice site	Exon length (bp)	5′ splice site	Size of intron (kb)	Accession number
1	CTTTGTGTTTATTAATGC**AG** ATAACAAG	224	AAAGTCCT **GT**GAGTTGAATTAGAAGGCT	?	Y14372
2	TTTATTTTTGTGTATTAC**AG** GCCTTTCG	139	TCCTCTCA **GT**ATGTCAGTTTTGGAGAGT	3.2	Y14373
3	CACATTTTTTGCTCTTTT**AG** GTCTTGGC	152	ATATCTCA **GT**GAGTTCTTCAGCTTTCAA	0.95	Y14374
4	CCTGTTGTTTTTTTTTCC**AG** GTAAGAAT	81	CCAATTTG **GT**ATAATTTTCCATTCCTGT	1.2	Y14375
5	GTCCTTCTGATTTTTTCC**AG** GTGAAAGT	150	CAGCAGCT **GT**AAGTCAATGAAAGTCCAA	0.5	Y14376
6	GTATGGGCCAAACCAAAT**AG** GTATCCTC	144	GTTGTAAG **GT**ATGTAGTAGAGTTTGTCT	0.111	Y14377
7	CTGTGGGCATTCTATTTT**AG** GTGCTGGT	99	AGATTCAG **GT**AGGAAACATTATTCCCCT	2.5	Y14377
8	TATTTTACCATTGATTAC**AG** GTGCAGGC	183	ACAACTTG **GT**AGGTTGCAAAATTGAAGT	1.75	Y14378
9	GTGGCTTTTCCCTCCCTT**AG** GGGTATAG	258	AAATCACG **GT**ATGCCAGTATTTTATGTA	1.45	Y14379
10	GAAAATTTTTGATTCTTT**AG** TGGATGAA	120	GAAATAAG **GT**AAGACATGGTCTTCTATA	0.4	Y14380
11	TGAACCGTTTTTCAATTC**AG** ATTCGGGA	87	CACCTTCT **GT**AAGTTTATGAAGTAGTAT	3.6	Y14381
12	TTTTATTTTTTCTTTGTA**AG** ACTATGCA	124	CCTAGAAG **GT**TAGTTAATCTTTACATTC	1.7	Y14382
13*	ACTAAACTCTTATCCAAC**AG** ATGCTGAG	96	ATTAGAAG **GT**ACTCAATGTAATTTCCA	1	Y14383
14	AAAACCCTCCCCTTTCGT**AG** AAAACCAT	(293)°	stop		Y14384

* Alternative spliced exon.
° Coding sequence length.

ney, brain, lung and heart tissues showed both the unprocessed and the processed product (data not shown). These results are consistent with those reported by Tripodi (14), which detected an analogue in-frame insertion of 96 nt in rat Add3b transcript expressed in several tissues. However the presence of differential RNA processing system for ADD3 as for ADD1 and ADD2 (21, 17) requires an elucidation about functional differences of the various molecular forms of this protein in different tissues.

Figure 1 shows that amino acid identity between human and rat ADD3 is 91.9% and the insertion of 32 amino acids observed both in rats and in humans (boxed) is located in the same position (85% homology). A very high homology between human and rat adducin was already seen for α and β subunits (12, 13). This correspondence suggests that both genes could present the same exon-intron organization. It is also clear that forces have tended more toward conservation of globular domain than the tail domain.

The comparison among exon sizes of human ADD1, ADD2 and ADD3 suggests the presence also in ADD3 of at least one exon upstream the translation start in 5′ UTR region (Table 2). The highest homology in exon sizes is among 5′ coding regions corresponding to N-terminal globular domain (protease resistant) and neck domains of adducin molecule. The most variable region is the C-terminal tail domain (protease sensitive), that exhibits localized similarities to the MARCKS proteins and contains most of the phosphorylation sites and the binding site for calmodulin required for association with spectrin and actin (22). Moreover, the conservation of most exon sizes among the three subunits is a further demonstration that

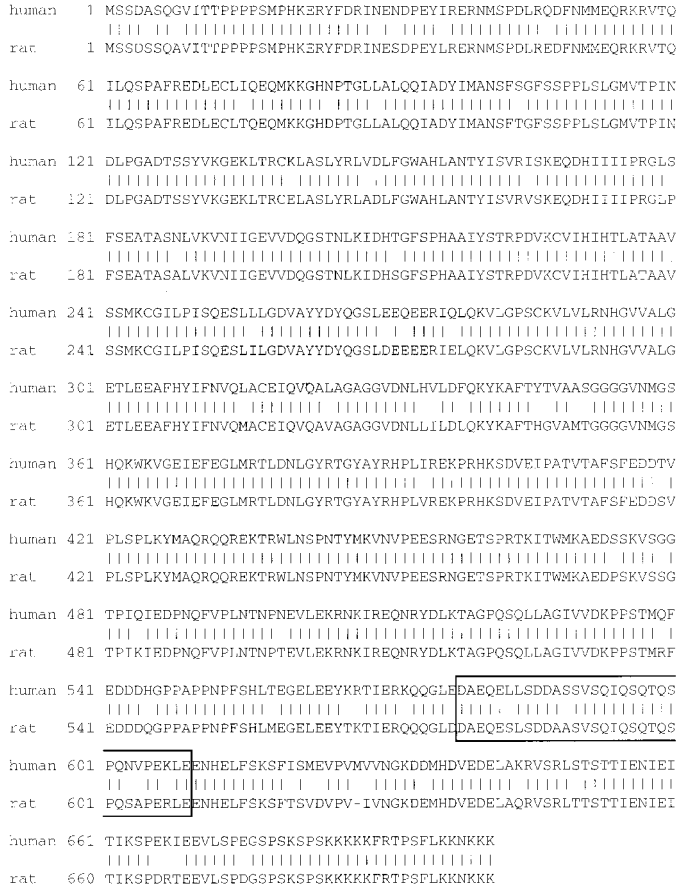

FIG. 1. Amino acid sequence alignment of the human and rat ADD3 genes. Identical residues are shown with a vertical bar (|). Boxed sequences represent the translated alternative spliced exon presents in both species.

TABLE 2

ADD1, ADD2, and ADD3 Exon Sizes

ADD1	ADD2	ADD3
—	312 (1)	—
132 (1)	119 (2)	—
215 (2)	217 (3)	224 (1)
163 (3)	139* (4)	139* (2)
152° (4)	152* (5)	152°* (3)
81° (5)	81* (6)	81°* (4)
150° (6)	6* (7a)	150°* (5)
—	144* (7b)	—
144° (7)	144* (8)	144°* (6)
99° (8)	99* (9)	99°* (7)
177 (9)	177 (10)	183 (8)
252 (10a)	258* (11)	258* (9)
93 (10b)	—	—
102 (11)	95* (12a)	120* (10)
—	24* (12b)	—
90 (12)	90 (13a)	87 (11)
—	717 (13b)	—
157 (13)	148 (14)	124 (12)
—	86 (15)	—
99 (14)	129 (16)	96 (13)
34 (15)	—	—
1892 (16)	1579 (17)	>293 (14)

Note. ADD1 exon sizes are from Lin (21) and the ADD2 from Gilligan (17). ADD1, ADD2, and ADD3 exon sizes are aligned according to initiation of translation. The exons identical in sizes are indicated by ° for ADD1 and ADD3 and by asterisk for ADD2 and ADD3.

ADD1, ADD2 and ADD3 belong to the same protein family.

A more precise localization of ADD3 gene was also undertaken by submitting the ADD3 sequence to the STS database. The tagged sequence and consequently human ADD3 gene has been mapped by screening the Genbridge 4 radiation hybrid panel at 551.35 centiRadios from top of chromosome 10 linkage group between markers D10S543 and WI-4132 (150 cM on the Généthon map), correspondent to 10q24.1–10q24.2. The homologous rat gene has been localized on chromosome 1q55 (14).

By the time a cDNA sequence called Adducin-like (ADDL) has been published by Katagiri (16), Genbank accession number D67031. It contains an ORF concordant in nucleotide and deduced amino acid sequence to our sequence, even if lacking the insertion of 32 aa, and a longer 3′ UTR region than U37122. ADDL cytogenetic localization is 10q24.2–10q24.3 in accordance to our Radiation Hybrid mapping. These data suggest that ADD3 and ADDL could represent the same gene.

In conclusion, our results indicate that: (1) the human ADD3 gene is composed at least of 14 exons and 13 introns spanning over 20 kb; (2) there is evidence of an alternative splicing event at 3′ end of the gene corresponding to the tail domain of the protein, which is the most variable region in all the three subunits; (3) human ADD3 amino acid sequence presents 91.9% of identity compared to rat sequence.

Characterization of the human ADD3 gene provides an important tool for developing the reagents necessary for mutation detection based on genomic DNA. A spontaneous polymorphism in the α-adducin gene is involved in genetic alterations of renal Na^+ transport and in the pathogenesis of primary hypertension in rat and human. Polymorphisms in the γ subunit could "per se" contribute to the determination of hypertension or could epistatically interact with the known polymorphism in the α subunit or with polymorphisms in other candidate genes (23).

REFERENCES

1. Hughes, C. A., and Bennett, V. (1995) *J. Biol. Chem.* **270,** 18990–18996.
2. Matsuoka, Y., Hughes, C. A., and Bennett, V. (1996) *J. Biol. Chem.* **271,** 25157–25166.
3. Barber, B. R., Ferrari, P., and Bianchi, G. (1994) *in* Handbook of Hypertension (Ganten, D., and de Jong, W., Eds.), pp. 316–45 Elsevier, Amsterdam.
4. Ferrandi, M., Tripodi, G., Salardi, S., Florio, M., Modica, R., Barassi, P., Parenti, P., Shainskaya, A., Karlish, S. J. D., Bianchi, G., and Ferrari, P. (1996) *Hypertension* **28,** 1018–1025.
5. Bianchi, G., Tripodi, G., Casari, G., Salardi, S., Barber, B. R., Garcia, R., Leoni, P., Torielli, L., Cusi, D., Ferrandi, M., Pinna, L. A., and Baralle, F. E. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 3999–4003.
6. Tripodi, G., Valtorta, F., Torielli, L., Chieregatti, E., Salardi, S., Trusolino, L., Menegon, A., Ferrari, P., Marchisio, P. C., and Bianchi, G. (1996) *J. Clin. Invest.* **97,** 2815–2822.
7. Casari, G., Barlassina, C., Cusi, D., Zagato, L., Miurhead, R., Righetti, M., Nembri, P., Amar, K., Gatti, M., Macciardi, F., Binelli, G., and Bianchi, G. (1995) *Hypertension* **25,** 320–326.
8. Cusi, D., Barlassina, C., Azzani, T., Casari, G., Citterio, L., Devoto, M., Glorioso, N., Lanzani, C., Manunta, P., Righetti, M., Rivera, R., Stella, P., Troffa, C., Zagato, L., and Bianchi, G. (1997) *Lancet* **349,** 1353–1357.
9. Castellano, M., Barlassina, C., Muiesan, M. L., Beschi, M., Cinelli, A., Rossi, F., Rizzoni, D., Cusi, D., and Agabiti-Rosei, E. (1998) *J. Hypertens.* **15,** 1707–1710.
10. Manunta, P., Cusi, D., Barlassina, C., Righetti, M., Lanzani, C., D'Amico, M., Buzzi, L., Citterio, L., Stella, P., Rivera, R., and Bianchi, G. (1998) *Kidney Int.* **53,** 1471–1478.
11. Ferrandi, M., Salardi, S., Tripodi, G., Barassi, P., Barisoni, L., Rivera, R., Manunta, P., Karlish, S., Ferrari, P., and Bianchi, G. (1999) *Am. J. Physiol.* **277,** in press.
12. Joshi, R., Gilligan, D. M., Otto, E., McLaughlin, T., and Bennett, V. (1991) *J. Cell Biol.* **115,** 665–675.
13. Tripodi, G., Casari, G., Tisminetsky, S., Bianchi, G., Devescovi, G., Muro, A., Tuteja, R., and Baralle, F. E. (1995) *Gene* **166,** 307–311.
14. Tripodi, G., Szpirer, C., Reina, C., Szpirer, J., and Bianchi, G. (1997) *Biochem. Biophys. Res. Commun.* **237,** 685–689.
15. Dong, L., Chapline, C., Mousseau, B., Fowler, L., Ramsay, K., Steven, J. L., and Jaken, S. (1995) *J. Biol. Chem.* **270,** 25534–25540.
16. Katagiri, T., Ozaki, K., Fujiwara, T., Shimizu, F., Kawai, A.,

Okuno, S., Suzuki, M., Nakamura, Y., Takahashi, E., and Hirai, Y. (1996) *Cytogenet. Cell. Genet.* **74,** 90–95.

17. Gilligan, D. M., Lozovatsky, L., and Silberfein, A. (1997) *Genomics* **43,** 141–148.
18. Shyamala, V., and Ferro-Luzzi Ames, G. (1989) *Gene* **84,** 1–8.
19. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Plainview, NY.
20. Breathnach, R., and Chambon, P. (1981) *Annu. Rev. Biochem.* **50,** 349–383.
21. Lin, B., Nasir, J., McDonald, H., Graham, R., Rommens, J. M., Goldberg, Y. P., and Hayden, M. R. (1995) *Genomics* **25,** 93–99.
22. Joshi, R., and Bennett, V. (1990) *J. Biol. Chem.* **265,** 13130–13136.
23. Barlassina, C., Schork, N. J., Manunta, P., Citterio, L., Sciarrone, M. T., Lanella, G., Bianchi, G., and Cusi, D. (1999) *Kidney Int.*, in press.