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The human αE-catenin gene *CTNNA1*: mutational analysis and rare occurrence of a truncated splice variant

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Abstract

Abnormal expression of the αE-catenin protein, a component of the E-cadherin/catenin cell adhesion complex, is frequently observed in human cancer cells. An inverse correlation between αE-catenin expression and tumor malignancy can be of prognostic value. Mutations of the αE-catenin gene, *CTNNA1*, were described in several human cancer cell lines and were found to result in aberrant cell adhesion. We have developed a polymerase chain reaction/single-strand conformation polymorphism-based method for mutation analysis of this gene in human tumor DNA. This approach enabled us to identify several polymorphisms in a set of desmoid tumors, demonstrating that this method is suitable for αE-catenin mutational analysis. On the basis of our genomic characterization data, we found that the previously reported alternative splicing of the αE-catenin gene actually generates a frame-shift, resulting in a truncated αE-catenin protein. This finding is unlike the other α-catenin family members αN-catenin and vinculin, which show in-frame alternative inserts. Furthermore, real-time quantitative reverse transcriptase–PCR analysis did not reveal relevant expression levels of this alternatively spliced αE-catenin variant neither in any human tissue or cell line tested, nor at any mouse developmental stage tested. Thus, contrary to previous notions, alternative splicing with in-frame insertion nearby the C-terminal end of the protein is not a general feature for all members of the α-catenin/vinculin family. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Cell adhesion; Adherens junction; αE-catenin; Cancer; Polymerase chain reaction/single-strand conformation polymorphism; Alternative splicing; Truncated protein

1. Introduction

The αE-catenin protein is localized at the zonula adherens (also called adherens junction) of simple epithelia. It forms part of the E-cadherin/catenin multiprotein complex and is essential to reach full functionality of the adhesion junction. In this complex the catenins interact with the transmembranous cell–cell adhesion molecule E-cadherin. Either β-catenin or plakoglobin (γ-catenin) binds to a carboxyterminal cytoplasmic domain of E-cadherin. α-Catenin then forms the bridge between the former catenins and the actin cytoskeleton by direct interaction with filamentous F-actin or through interaction with either vinculin or α-actinin [1].

The linkage of E-cadherin to the actin cytoskeleton seems to be crucial for strong E-cadherin mediated cell–cell interaction. Several human invasive cancer cell lines were described to show loose cellular adhesion due to αE-catenin inactivating mutations [2–6]. For these cell lines, a restored cell adhesion was observed upon transfection of α-catenin cDNAs, which resulted in increased cell aggregation and decreased cell growth rate. Recently, precancerous hyperproliferation and sustained activation of the Ras-MAPK cascade was found in mouse skin with tissue-specific ablation of the αE-catenin gene [7]. Also in human tumors, abnormal α-catenin expression is frequently observed. It occurs either simultaneously with the loss of E-cadherin expression [8] or independently. In the latter case it was described for some tumor types to have a more reliable prognostic value than loss of E-cadherin expression [9,10].

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Until now, three α-catenin isoforms were reported which show an overall identity at the amino acid level of 56%: αE-catenin, which is ubiquitously expressed, mainly in epithelial tissues [11], αN-catenin whose expression is restricted to neural tissues [12,13] and αT-catenin which is mainly expressed in heart tissue [14]. These α-catenins are also structurally and functionally related to vinculin, a protein expressed in focal adhesions [15]. For both αE- and αN-catenin and for vinculin, an alternative splice variant with an in-frame insert nearby the C-terminal end was reported [13,16,17]. For αE-catenin, the longer splice variant was called αE2-catenin, the shorter one αE1-catenin. Another α-catenin-vinculin family member, α-catulin, also shows alternative splicing but this leads to C-terminal truncation of the protein [18].

Here we report on the genomic structure, the exon-flanking intron sequences and a polymerase chain reaction/single-strand conformation polymorphism (PCR/SSCP)-based mutation analysis method developed for the human αE-catenin gene *CTNNA1*. We also found that one of the two alternatively spliced forms of αE-catenin is rarely expressed and results in an unreported C-terminal truncation of the protein.

2. Materials and methods

2.1. Isolation and DNA sequencing of exon-flanking intron sequences

Pools of a human BAC library (Genome Systems, St. Louis, MO), a P1 library and a PAC library (UK HGMP Resource Centre, Hinxton, Cambridge, UK) were screened by PCR reactions specific for intron 1–intron 2, intron 2–exon 3 or exon 13–intron 13 boundaries, respectively. One BAC clone (BAC97N15), one P1 clone (#14069) and three PAC clones (PAC14G18, PAC132G19, PAC147B1) were obtained. DNA was sheared by controlled sonication and fragments of about 700 bp were purified by agarose gel electrophoresis, blunted with T4 DNA polymerase and Klenow polymerase and subcloned in the pUC18 vector. Colonies were hybridized with a radioactively labeled αEctn-probe1, αEctn-probe2 or exon-specific primers [19] and clones of interest were sequenced. Genomic fragments containing all exons were identified. Sequences were analyzed using DNAstar (DNAstar, Madison, WI) and Staden software [20].

2.2. SSCP analysis of tumor samples

For SSCP, PCR reactions were carried out in a volume of 25 μl containing 100 ng genomic DNA, 2–4 mM $MgCl_2$, 12.5 pmol of each primer, 200 μM dATP, dGTP and dTTP, 20 μM dCTP, 0.5 μCi [α-^{32}P]dCTP (3000 Ci/mmol; Amersham Pharmacia Biotech, Rainham, UK) and the buffer supplied with the *Taq* DNA polymerase (Life Technologies, Paisley, UK). Tumor and non-tumor DNAs were obtained from patients with sporadic desmoid tumors as described before [21]. Primers for the αE-catenin gene were designed using the Oligo 5.0 (National Biosciences, Plymouth, MN) software. A total of 35 cycles (30 s at 94°C, 30 s at 55–60°C and 1.5 min at 72°C) were programmed in a thermal cycler (GeneAmp PCR System 9600, PE Applied Biosystems, Foster City, CA). Annealing temperatures used for each amplicon are indicated in Table 1. Five μl of PCR product was added to 5 μl formamide dye (95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol). The mixture was heated at 99°C for 2 min and snap-cooled on ice. Two μl was loaded on two different vertical non-denaturing polyacrylamide gels (5% polyacrylamide with 5% cross-linking; Sequi-gen sequencing cell, BioRad, Richmond, CA) either containing 5% glycerol (run at room temperature), or lacking glycerol (run at 8°C). Electrophoresis was performed overnight us-

Table 1
Primers used for PCR/SSCP mutation detection of the αE-catenin gene *CTNNA1*

Exon no.	Forward primer sequence	Reverse primer sequence	Amplicon size (bp)	Annealing temp. (°C)	[$MgCl_2$] conc. (mM)
2	GATGTTTGCCTGACTGACTT	ATAGAACCATGTTACAATGT	185	60	2.5
3	AACTTAATCTTGCTGTCTTTA	CCTGCCTCTCTTTACCTAA	312	55	4.0
4	CAGTTATTTGAGTTCATTCT	GTAGCAATCTTCCTGTGATA	271	55	2.5
5	ATGCCATCTTCTTTACAGAC	AGAATGAGTTTTTCCCTCTA	252	60	2.5
6	AGTGCTCCAATTTCTTGTTT	TATCTTTTGGGGGATGGTCA	358	60	2.5
7	AAGAAGGGAACAGAGATGA	TCCATAAATATCTTACTTCA	295	55	4.0
8	GCTATCATTAGGTTTCTTTGTA	AAATTGAGAACTTAGAAAAACA	187	55	2.5
9	AGCAATCTATTTAATACCTT	ATTAAAACTCACCACACTTC	277	55	2.5
10	TTTCCTTAGCAGTCAAAAGA	CAAAATAAACCTCATCTCCA	222	55	2.5
11	GACAAAGCCATGACTCTGAA	TTGCCCATTAATACAACTTA	287	55	2.5
12	TGCCAGCTTACAGTTGCCACC	TGTGTGCGAGCACTGGAAAGG	307	60	2.5
13	GGGGAATGATGCTGCCTGCTG	TGACGCATTGCTCAAGTTTAG	276	60	2.5
14	AATGCTGAGTGATTTTGACTT	AAACGGTGTTCTGAATACTGAG	372	60	2.0
15	TTGGCTAATGCACTCTGAGAA	GACCTGTGGGGAAGAAAGTAG	307	60	2.5
16	TGGGGCTTTGTGGACAATCTT	GACCCACCTGGCAAGTCTG	219	55	2.5
17	TCAGAAACAGCCGTGGGGTCG	CACTGGCCCTCTTTTTGTTCA	242	60	2.5
18	GTCAGGCCGGTGCTTCTTAC	ATTTGAGTGACGAACAGTGT	395	60	2.5

ing 0.5× TBE at 3 W. The gels were transferred onto Whatman 3MM paper, dried and visualized by exposing the gel to a PhosphorImager screen (Molecular Imager, FX, BioRad). When bands needed to be further analyzed by DNA sequencing, bands were excised from the Whatman paper and placed in 150 μl H_2O. Incubation of the excised bands for 30 min at 60°C followed by 15 min at −70°C was repeated three times. A 10-μl aliquot was used as a template for a non-radioactive PCR reaction. Subsequently, the PCR product was purified using the CONCERT PCR Purification kit (Life Technologies) and sequenced (PE Applied Biosystems).

2.3. Cell cultures

Most of the cell lines used were from the American Type Culture Collection (ATCC, Rockville, MD): colon adenocarcinoma cell line Colo320DM (CCL220), leiomyosarcoma cell line SK-LMS-1 (HTB-88), epidermoid carcinoma cell line A431 (CRL-1555), SV40-transformed lung fibroblasts WI-38-VA13-subline 2RA (CCL75.1, abbreviated below as VA13), breast cell line HBL-100 (HTB-124). GLC34 is derived from a small cell lung carcinoma [22]. Cell line LICR-HN3 is derived from a head squamous cell carcinoma [23]. WRO and B-CPAP are thyroid carcinoma cell lines [24,25]. MCF-7/AZ is derived from the MCF-7 (HTB-22) mammary carcinoma cell line [26]. MKN45 is a gastric carcinoma cell line and KATO-III is a signet ring cell carcinoma cell line [27]. FS4 is a human foreskin fibroblast cell line.

2.4. Reverse transcriptase–polymerase chain reaction (RT–PCR) analysis of αE-catenin splice variants.

Total RNA was extracted from different human cell lines and different mouse developmental stages using the RNeasy kit (Qiagen, Chatsworth, CA). Quality of RNA samples was checked by agarose gel electrophoresis. RNA from human tissues was purchased from Clontech (Clontech Laboratories, Palo Alto, CA). Reverse transcription of cDNA was done using the Reverse Transcription Reagents kit (PE Applied Biosystems) with random hexameric primers according to the manufacturer's instructions. Primers and probes for the quantitative PCR were designed using the Primer Express software (PE Applied Biosystems). The following primer pairs were used: P1 (5′-gcggggagcttgttgtct-3′) plus P2 (5′-aagttcttggctgcctggat-3′) for human αE1-catenin, and P3 (5′-ggcactgcaagggctgaa-3′) plus P2 for human αE2-catenin. Probes used were PrA (5′-FAM-catggcgctgtccacccca-TAMRA-3′) for αE1-catenin and PrB (5′-VIC-ctccccttgctggccactcactgg-TAMRA-3′) for αE2-catenin. To detect the mouse αE- and αN-catenin splice variants the following primer pairs were used: 5′-tctctactgccaccagctcaac-3′ and 5′-aagccatcccctgtgacttct-3′ for αE-catenin, and 5′-ctaccttcagcggattgctttg-3′ and 5′-ttgacagctgctgttccatagac-3′ for αN-catenin.

Primers were purchased from Life Technologies and probes were from PE Applied Biosystems or Biosource International (Camarillo, CA).

For real-time quantitative RT–PCR, the absolute standard curve method was used in order to determine expression levels of both splice variants of αE-catenin. To this end, PCR products P1–P2 and P3–P2 were cloned in the pGEMT-easy vector (Promega, Madison, WI) and transcribed using SP6 polymerase (Promega). Concentration of RNA was measured at 260 nm and converted to the number of copies on the basis of the RNA size. A 4-fold serial dilution of this in vitro transcribed RNA was made in yeast RNA. All samples were reverse transcribed and the corresponding Ct (=cycle threshold) values were determined. Finally the standard curve was constructed by plotting log starting copy number versus Ct value.

All PCR reactions were performed using an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). A master mix was prepared on ice with 1× Taqman Buffer, 2.5 or 3.5 mM $MgCl_2$ (for the αE2- and αE1-catenin specific reactions, respectively), 200 μM of dATP, dCTP and dGTP, 400 μM dUTP, 200 nM of each primer, 100 nM probe, and 1.25 units of AmpliTaq Gold DNA polymerase (PE Applied Biosystems). Five μl of each appropriately diluted reverse transcriptase sample were added to 45 μl of the PCR master mix. The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. Experiments were performed in triplicate. The αE1- and αE2-catenin mRNAs in each sample were quantified by measuring the Ct value and by using the absolute standard curves to determine the starting target message quantity. Finally, the ratio αE1-catenin/αE2-catenin was determined.

3. Results and discussion

3.1. Mutational analysis of the human αE-catenin gene CTNNA1

Sixteen exon–intron boundaries of the human αE-catenin gene have been described by Furukawa et al. [28]. Exon-flanking intron sequences were however not determined. Therefore, P1, BAC and PAC genomic libraries were screened by PCR to isolate genomic fragments containing the αE-catenin gene. Exons were subcloned from these genomic clones and sequenced. As compared to the results of Furukawa et al. [28], we found an additional intron within the previously described exon 2 (Fig. 1).

The exon-flanking intron sequences were used to design PCR/SSCP primers that enable amplification of all coding exons (see Table 1) and subsequent mutational analysis. Twenty-four desmoid tumors were analyzed for αE-catenin sequence alterations by SSCP analysis of the PCR products. Five polymorphisms were found (Table 2).

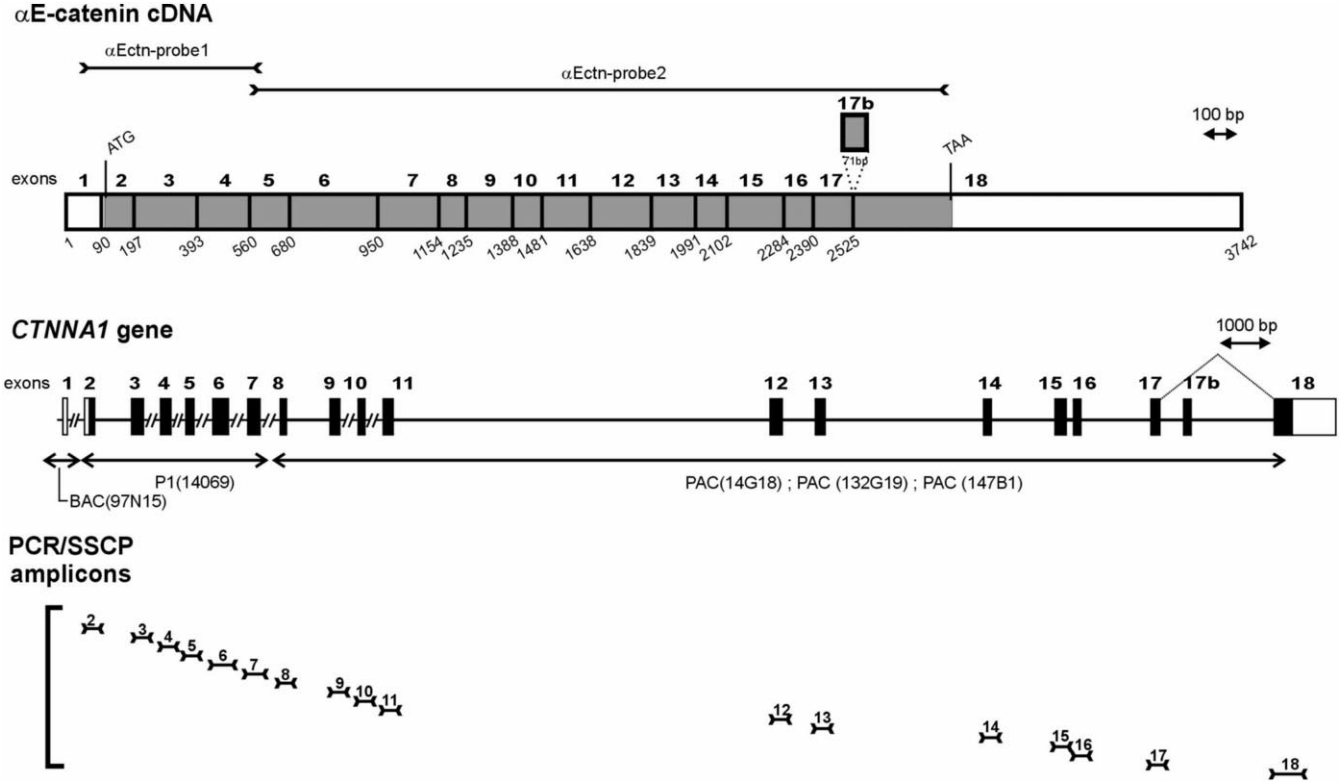

Fig. 1. Molecular cloning and genomic organization of the human αE-catenin gene *CTNNA1*. The αE-catenin cDNA sequences with the corresponding 18 exons and the alternatively spliced exon 17b are as indicated. White boxes indicate 5′ and 3′ untranslated region sequences. The specific probes αEctn-probe1 and αEctn-probe 2 are indicated at the top. The human *CTNNA1* gene is also shown schematically. The P1, PAC and BAC genomic clones, from which the various exons were isolated, are indicated below the gene. The PCR amplicons used for genomic SSCP are indicated at the bottom. All exon sequences have been deposited with the public databases (accession nos. AF102786–AF102803).

They were located within exon 5, intron 9, intron 15 and exon 16 (twice). One more sequence alteration in exon 6 was found in tumor no. 27 and turned out to be a missense mutation of αE-catenin: Gln206His (Fig. 2A). As this αE-catenin sequence alteration is not within the β-catenin-binding domain, it is unlikely that this mutation can explain the strong nuclear β-catenin staining found in these tumors [21]. This sequence alteration was also found in the germline DNA of the patient. However, no familial cancer predisposition was observed within the family of this patient, and we also found the same sequence alteration in the DNA from his healthy 83-year-old mother (Fig. 2B). It is therefore unlikely that this amino acid substitution does change the functional properties of αE-catenin protein dramatically or that this predisposes to (desmoid) tumor formation.

By this αE-catenin mutation analysis it was demonstrated that our mutation screen is suitable to search for tumor-confined αE-catenin sequence alterations. In the future, the many different tumor types showing abnormal αE-catenin expression patterns will be screened for such mutations.

3.2. *Alternative splicing of the human αE-catenin gene CTNNA1*

The alternatively spliced exon (17B), as described by Rimm et al. [16], was found 175 bp downstream of the splice donor site of exon 17 (Fig. 1). Sequence alignments showed however that this exon comprises 71 bp instead of the previously reported 72 bp. Our results are supported by recent sequence data for *CTNNA1* in the htgs database

Table 2
Polymorphisms detected in the human αE-catenin gene *CTNNA1*

Exon no.	Codon no. or intron position	Allele 1	Allele 2	Frequency of allele 2
5	Leu (180)	CTA	CTC	2/22
9	Intron 8 at 8 nt	CTGGA	CTCGA	9/21
15	Intron 14 at 67 nt	TTCCA	TTACA	1/23
16	Ser (740)	TCG	TCA	10/22
16	Val (742)	GTC	GTG	2/22

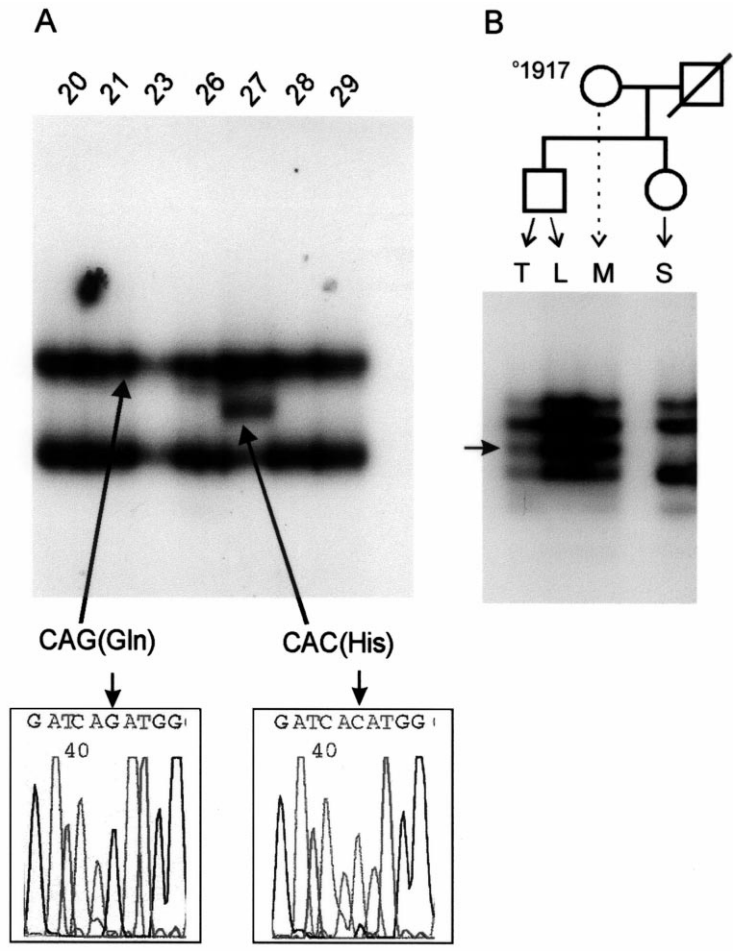

Fig. 2. Results of SSCP analysis of exon 6 of the *CTNNA1* gene for several desmoid tumor samples. (A) Fragment of an SSCP gel representing the exon 6 analysis of seven tumors. DNA sequence analysis showed that the shifted band represents a CAG to CAC sequence alteration changing αE-catenin codon no. 206 from Gln into His. (B) The same band-shift (arrow) is observed for leukocyte DNA (L) of the tumor patient and for leukocyte DNA of the patient's mother (M; born in 1917), but not for leukocyte DNA of the patient's sister (S). No DNA was available from the deceased patient's father.

(GenBank accession number AC011405) and by sequences present in the human EST database (not shown). Furthermore, translation of the cDNA sequence of αE2-catenin, which includes exon 17B, resulted in a frame-shift and in truncation of the predicted carboxy terminal part of the protein (Fig. 3A). Moreover, as the carboxy-terminus comprises an F-actin binding motif required for strong state adhesion activity [29], this alternative splicing is likely to interfere with F-actin binding and with the functionality of the αE-catenin protein [30]. To test if this alternatively spliced transcript is significantly expressed to become biologically relevant, we performed real-time quantitative RT–PCR analyses detecting either one of both splice variants (Fig. 3B). To avoid amplification of contaminating genomic DNA, each primer of a pair was placed in a different exon per reaction. Sets of primers and probes were chosen so that only one spliced form could be detected. We found that in all tissues examined, the alternative transcript is present in much lower abundance than the αE1-catenin transcript (Table 3). The expression level of αE2-catenin transcripts was often at the detection limit

Table 3
Expression levels of the two αE-catenin splice variants in different cell lines and tissues

Cell line	αE1-catenin/αE2-catenin[a]
KATOIII	4134
MKN45	1658
HBL100	3803
MCF-7/AZ	17268
FS4	14852
VA13	10773
B-CPAP	4755
WRO	6579
LICR-HN3	7987
GLC34	6542
A431	13331
Colo320DM	29145
SK-LMS-1	10544
Tissue	
Lung	97523
Kidney	63013
Breast	85013
Heart	No αE2-catenin detectable

[a]The ratio values given are the average of two independent real-time quantitative RT–PCR experiments.

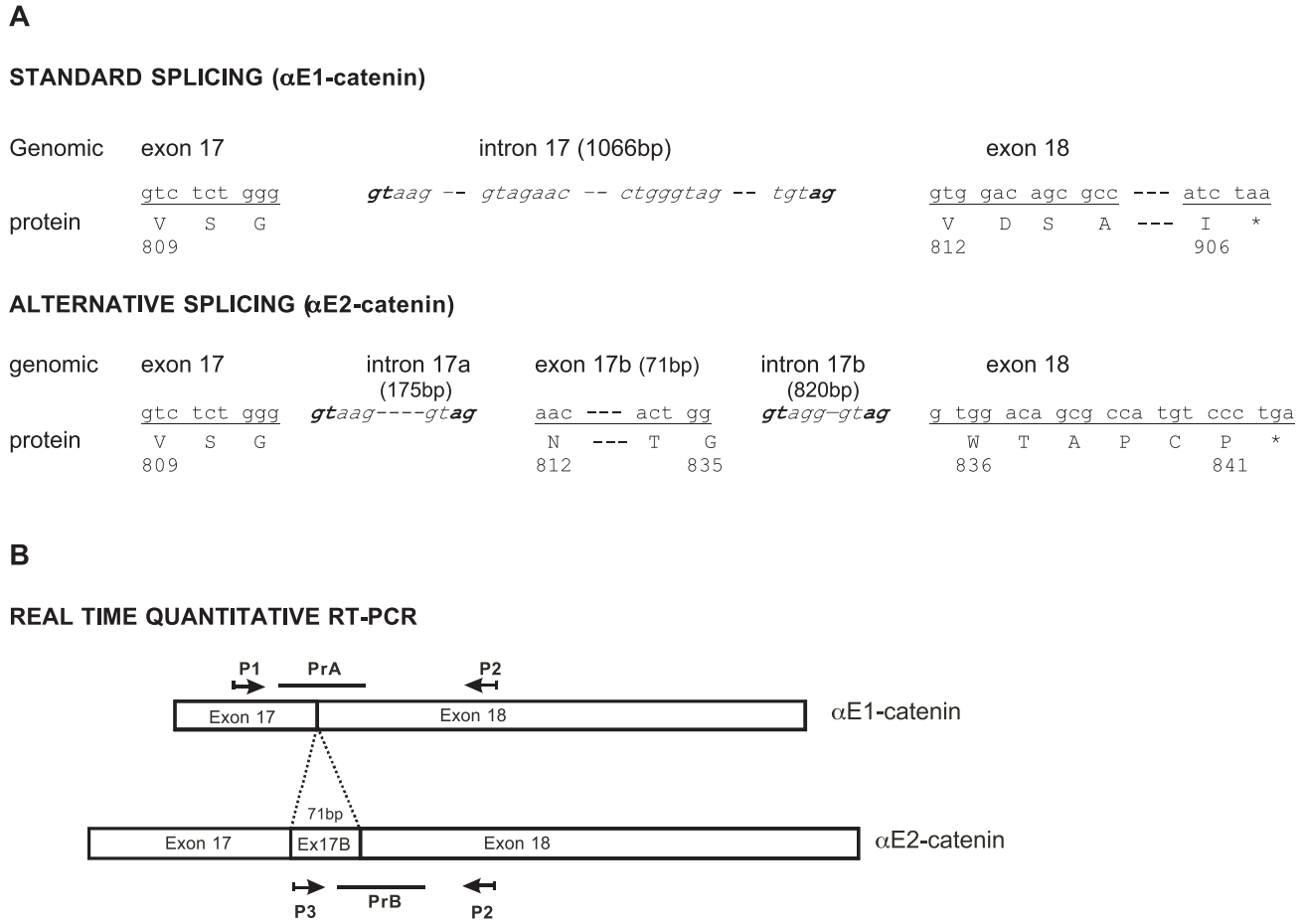

Fig. 3. Alternatively spliced human αE-catenin mRNA encodes the αE2-catenin isoform. (A) Schematic overview of the alternative splice acceptor sequence within intron 17, the resulting frame-shift and the shortened ORF in the alternative isoform αE2-catenin. Protein-encoding genomic sequences are underlined. Intronic sequences are in italics (bold for splice consensus sequences). Codons, some of which are numbered, are in one-letter abbreviations. (B) Schematic representation of the real-time quantitative RT–PCR reactions; the positions of the primers (P1, P2, P3) and the probes (PrA, PrB) are as indicated.

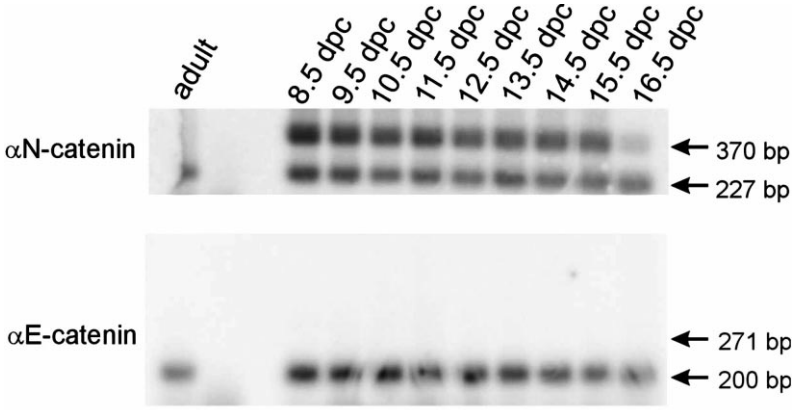

Fig. 4. Expression of αN- and αE-catenin splice variants during specific stages of mouse embryonic development. RT–PCR using primers flanking the region of alternative splicing and subsequent hybridization with a specific probe reveals the two predicted products for αN-catenin, while for αE-catenin only the product derived from the shorter splice variant is detected. The developmental stages from which the RNAs were derived are as indicated. Dpc, days post coitum.

of the system. For heart tissue, we could not detect any αE2-catenin splice form at all. To test whether this alternatively spliced variant is expressed during a specific stage of embryonic development, we performed an RT–PCR analysis on RNAs from different mouse developmental stages. We could readily detect the two splice variants of αN-catenin in each stage; however, for αE-catenin we could detect only the shorter αE1-catenin (Fig. 4).

Our results are in contradiction with those of Rimm et al. [16]. These authors reported that the αE2-catenin isoform is widely expressed, and that its abundance roughly correlates with the level of non-epithelial tissue. We found however almost no expression of the alternative splice variant. Also in the non-epithelial cell lines tested (FS4, VA13 and SK-LMS-1), αE2-catenin expression levels were low.

A similar alternative splicing resulting in C-terminal truncation of the protein was found for α-catulin, but also there the alternative α2-catulin has a far less abundant expression [18]. Two other α-catenin-vinculin family members, namely vinculin and αN-catenin, can be expressed as isoforms with in-frame inserts nearby their C-terminal ends [13,17]. The vinculin alternative splice variant, the so-called meta-vinculin, contains an in-frame insert of 68 amino acid residues that positively influences binding of vinculin to actin [1]. Functional differences between the two αN-catenin splice variants are so far not known. In conclusion, it is noteworthy that, contrary to previous reports, alternative splicing with in-frame inserts nearby the C-terminal end of the protein is not a general feature for all members of the α-catenin/vinculin family.

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