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Identification of Alternative Splice Products Encoded by the Human Coxsackie-Adenovirus Receptor Gene

Inge Thoelen,* Caroline Magnusson,† Sven Tågerud,† Charlotta Polacek,† Michael Lindberg,† and Marc Van Ranst*,[1]

**Laboratory of Clinical & Epidemiological Virology, Department of Microbiology and Immunology, Rega Institute for Medical Research, University of Leuven, Leuven, Belgium; and †Department of Chemistry and Biomedical Sciences, University of Kalmar, Kalmar, Sweden*

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The human cellular receptor for group B coxsackieviruses and adenoviruses (HCAR) is a transmembrane glycoprotein which belongs to the immunoglobulin superfamily. We describe alternative splicing of the HCAR-gene and the existence of three exon-skipping splice variants in addition to the originally identified seven exon-encompassing mRNA transcript. Expression of the splice variants theoretically results in truncated proteins, possibly leading to impaired viral binding and/or the occurrence of soluble viral receptors due to the absence of the transmembranous region. Consequently, this could markedly influence the efficacy of an adenovirus subgroup C-mediated gene therapy.

Key Words: **HCAR; alternative splicing; immunoglobulin-like domains.**

Adenoviruses and group B coxsackieviruses are phylogenetically unrelated pathogens that bind to the same cell surface protein, known as the coxsackie-adenovirus receptor (CAR) (1–3). Adenoviruses are naked, icosahedral, double-stranded DNA viruses with fibers projecting from the penton subunits of the capsid, terminating in globular knob domains. They are divided into six subgroups (A–F) and cause a variety of childhood and adult diseases such as conjunctivitis, respiratory diseases, and gastroenteritis. The group B coxsackieviruses belong to the family of the Picornaviridae. They are small, naked, icosahedral, positive-stranded RNA viruses that are common and significant causative agents of upper respiratory infections, meningitis, encephalitis, and myopericarditis. Adenovirus infection as well as adenovector-mediated gene transfer is initiated by attachment of the viral fiber knob to HCAR, the primary high-affinity adenovirus receptor. Virus internalization into the target cells requires subsequent interactions between the viral penton base and cell surface $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (4–6). The mechanism by which picornaviruses enter cells or release infectious RNA into the cytoplasm is not yet fully understood. In contrast to adenoviruses, group B coxsackieviruses lack fiber structures and are presumed to bind their cellular receptor in a canyon shaped structure on the viral surface (7–9).

CAR is a 46-kDa integral membrane glycoprotein with an unknown cellular function that is expressed in a wide range of human and murine cell types. Honda *et al.* (10) recently described that the mouse homologue of human CAR, that shows more than 80% similarity with the human cDNA-sequence, may function naturally as a cell adhesion molecule in the developing mouse brain. *In vivo,* the receptor seems to be expressed preferentially in epithelial cells of multiple organs. The highest CAR-mRNA expression of mouse and rat was found in the liver, whereas the strongest expression of human CAR-mRNA was noted in the heart, pancreas, and brain (6, 9).

The gene that encodes HCAR is located on chromosome 21q11.2. It consists of seven exons that are distributed over an area of 54 kb and produce a 365-amino acid (aa) protein (11, 12). A signal peptide (19 aa) originates from exon 1; exon 2 and 3 constitute the Ig1 domain, exon 4 and 5 the Ig2 domain; the first part of exon 6 encodes the transmembrane region (21 aa), whereas the last part of exon 6 and 7 give rise to a highly conserved cytoplasmic tail (107 aa) (1, 2). The extracellular portion of the receptor (218 aa) consists of two immunoglobulin-like domains: the N-terminal Ig1 is related to the immunoglobulin V-fold and the more C-terminal Ig2 is related to the IgC2-fold (13). Structural analysis of the mechanism of adenovirus binding

[1] To whom correspondence should be addressed to at Laboratory of Clinical and Epidemiological Virology, Department of Microbiology & Immunology, Rega Institute for Medical Research, Minderbroedersstraat 10, BE-3000 Leuven, Belgium. Fax: 32-16-347900. E-mail: marc.vanranst@uz.kuleuven.ac.be.

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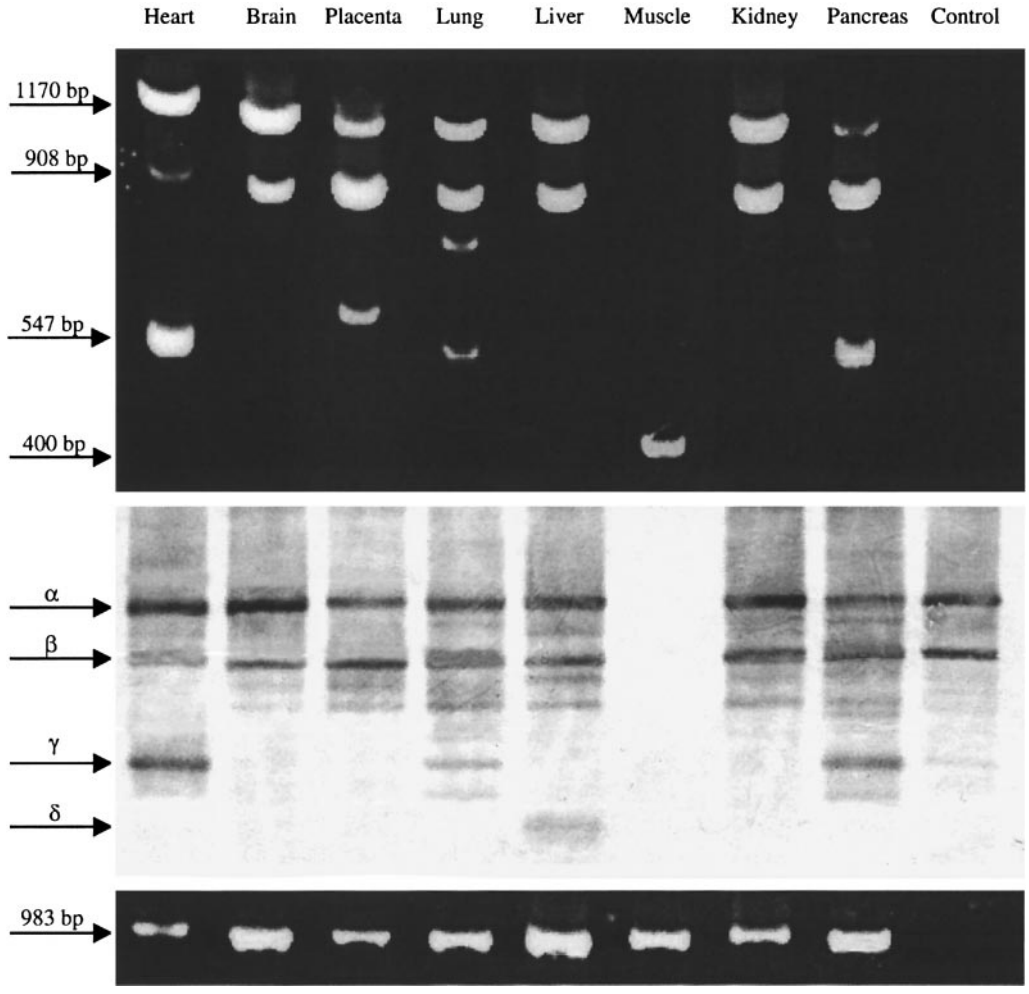

FIG. 1. Semi-nested PCR on a human multiple tissue cDNA-panel and Southern blot analysis. (*Top*) Gel electrophoresis after semi-nested PCR. The indicated bands of 1170, 908, 547, and 400 bp correspond to the α-, β-, γ-, and δ-variants, respectively. The control sample is a negative control. (*Middle*) Southern blot analysis of the semi-nested PCR-products with the HeLa RT-PCR product as a positive control. The α-, β-, γ-, and δ-variants are indicated. Other bands visible on the blot are due to heteroduplex formations. (*Bottom*) A control PCR with G3PDH-primers gives rise to a 983-bp -fragment in all tissues. The control sample is a negative control.

to HCAR revealed that only the Ig1 domain makes contact with the fiber knob. In contrast, molecular interactions of amino acid residues involved in attachment of group B coxsackieviruses to HCAR may reside in the Ig2 domain or in an overlap region between Ig1 and Ig2 (5, 9, 14).

Understanding the role of HCAR in the pathogenic process of both group B coxsackie-viruses and adenoviruses is important for the investigation of the biology and medical aspects of these viral infections. Also, adenovirus-based gene therapy requires thorough knowledge of receptor distribution and function. Gene therapy is an innovative therapeutic strategy for the treatment of benign and malignant diseases, based on introducing exogenous genes in target cells, to compensate for dysfunctional crucial genes. Recombinant human subgroup C adenoviruses (serotypes 2 and 5) are widely used as efficient vector delivery systems (15–17).

In this study we describe different aberrant splice products of HCAR mRNA transcripts which might influence virus attachment to the cell.

MATERIALS AND METHODS

RT-PCR on HeLa cell-RNA. Total RNA was extracted from HeLa cells using the RNeasy mini kit (Qiagen, Westburg, The Netherlands). The entire coding region of the HCAR-gene was amplified by a one-step RT-PCR (Qiagen OneStep RT-PCR kit, Qiagen, Westburg, The Netherlands) with 0.2 μg HeLa-RNA and two HCAR-specific primers (HCAR-F: 5′-AGGAGCGAGAGCCGCCTAC-3′ and HCAR-R1: 5′-ACGGAGAGCACAGATGAGACA-3′). The RT-PCR was performed in a 50 μl reaction volume containing 10 μl 5× Qiagen OneStep RT-PCR buffer, 400 μM of each dNTP, 2 μl enzyme mix (an optimized combination of Omniscript and Sensiscript reverse transcriptase and a HotStarTaq DNA Polymerase, Qiagen, Westburg, The Netherlands), 10 units RNase inhibitor (Perkin Elmer, Foster

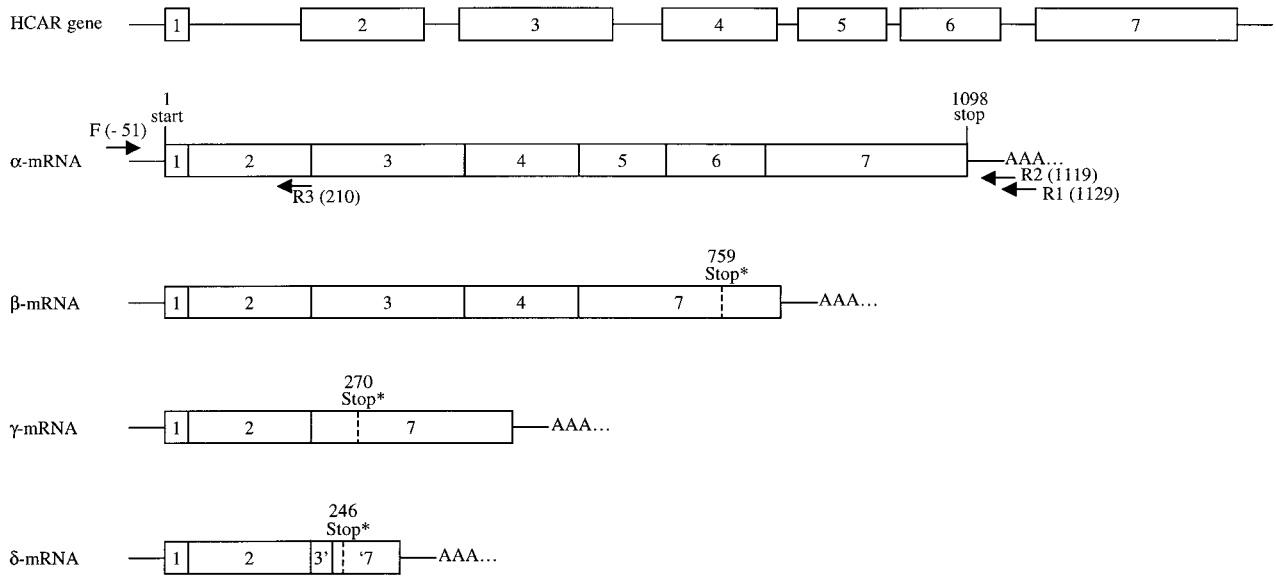

FIG. 2. Structure of the HCAR splice variants. Sequencing analysis shows three splice variants in addition to the normal transcript (α): the β-variant is an exon 5-6 skipping transcript, the γ-variant is an exon 3-4-5-6 skipping transcript and in the δ-variant exons 1-2, the first 21 nucleotides of exon 3 and the last 97 nucleotides of exon 7 are spliced together. F, R1, and R2 are the primers used for RT-PCR and semi-nested PCR. F and R3 are the primers used for HCAR-specific probe assembly. Stop* indicates the premature stop codons in the different splice variants.

City, CA), 0.6 μM of each primer, and RNase-free water to 50 μl. The reaction was carried out with an initial reverse transcription step at 54°C for 30 min, followed by PCR activation at 95°C for 15 min, 35 cycles of amplification (94°C, 30 s; 62°C, 30 s; 72°C, 1 min), and a final extension of 10 min at 72°C in a GeneAmp PCR System 9700 thermal cycler (Perkin Elmer, Foster City, CA). PCR-products were run on a 2% agarose gel, stained with ethidium bromide, and visualized under UV-light. Separated bands were isolated from the gel using the QIAquick Gel Extraction kit (Qiagen, Westburg, The Netherlands). Sequencing analysis of the PCR-products was performed using the ABI Prism BigDye Terminator Cycle Sequencing Reaction kit on a ABI Prism 310 DNA sequencer (Perkin Elmer Applied Biosystems, Foster City, CA), according to the manufacturer's instructions.

Semi-nested PCR on a human multiple tissue cDNA-panel. A panel of eight first-strand cDNA preparations from human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (Human MTC Panel 1, Clontech, Palo Alto, CA) was subjected to semi-nested PCR with primers HCAR-F and HCAR-R1 and subsequently HCAR-F and HCAR-R2 (5′-CAGATGAGACATATGGAGGCTC-3′). In both PCR-reactions, 1 unit Taq polymerase, 1 μl of a 25 mM dNTP-mix, 10 μl 5× buffer C (PCR Optimizer kit, Invitrogen, The Netherlands), and 5 pmole of forward and reverse primer were used. In the first PCR-reaction, 2 ng of the tissue cDNA were added as a template while in the subsequent semi-nested reaction 2 μl of the 50 μl product of the first PCR-reaction was used. The first amplification profile involved an initial denaturation at 94°C for 30 s, followed by a three-step cycle of 30 s at 94°C, 45 s at 55°C, and 1 min at 72°C for 47 cycles, and ending with a final extension of 5 min at 72°C. The semi-nested PCR reaction differed from the first profile in the initial denaturation step (94°C for 5 min), the annealing step (30 s at 56°C) and the number of cycles (35 cycles). The semi-nested PCR-products were subjected to polyacrylamide gel electrophoresis (9 μl PCR-product) and agarose gel electrophoresis (35 μl PCR-product). Separated bands were excised from the 2.5% agarose gel and purified with the QIAquick Gel Extraction kit (Qiagen, Westburg, The Netherlands) and analyzed by nucleotide sequencing as described above.

The cDNA preparations within the MTC panel have been carefully normalized to the mRNA expression levels of at least four different housekeeping genes. A control PCR with primers that amplify a 983-bp fragment of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) housekeeping gene (G3PDH-F: 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′; G3PDH-R: 5′-CATGTGGGCCATGAGGTCCACCAC-3′) was performed in parallel to verify that similar amounts of cDNA were provided in each preparation.

Southern blotting. 1/25 dilutions of the semi-nested PCR-samples and a 1/50 dilution of the HeLa RT-PCR product were run on a second 2.5% agarose gel and blotted to a positively charged nylon membrane (Roche Molecular Biochemicals, Germany) by capillary transfer with 20× SSC. The DIG-High Prime DNA labeling and detection starter kit I (Roche Molecular Biochemicals, Germany) was used to generate digoxigenin-labeled HCAR-specific probes (random-primed labeling), starting from a DNA-template (1 μg) corresponding to exon 1 and 2 of HCAR (DNA-fragment amplified with primers HCAR-F and HCAR-R3 (5′-CACTTGATCCACCTTCTGATTATC-3′) using 0.8 μg HeLa-RNA and the same one-step RT-PCR conditions as described above, except for the RT-temperature (52°C) and the annealing temperature (61°C)). After quantification of the labeling efficiency, hybridization was done overnight at 42°C in DIG Easy Hyb hybridization solution provided in the kit. Subsequently the hybridized probes were immunodetected with anti-digoxigenin-AP conjugate and visualized with the colorimetric substrates NBT/BCIP, according to the manufacturer's instructions. The DIG-labeled DNA molecular weight marker VI (Roche Molecular Biochemicals, Germany) was used for accurate molecular weight determination of the DIG-labeled hybrids.

Prediction of transmembrane helices. The Dense Alignment Surface (DAS) Transmembrane Prediction Server (www.biokemi.su.se/~server/DAS/; 18) and the PredictProtein Server (cubic.bioc.columbia.edu/predictprotein; 19) were used to search for putative transmembrane regions in the altered and truncated carboxy-terminal ends of the three isoforms. These tools predict whether the different HCAR isoforms might be expressed as membrane proteins or excreted as soluble forms.

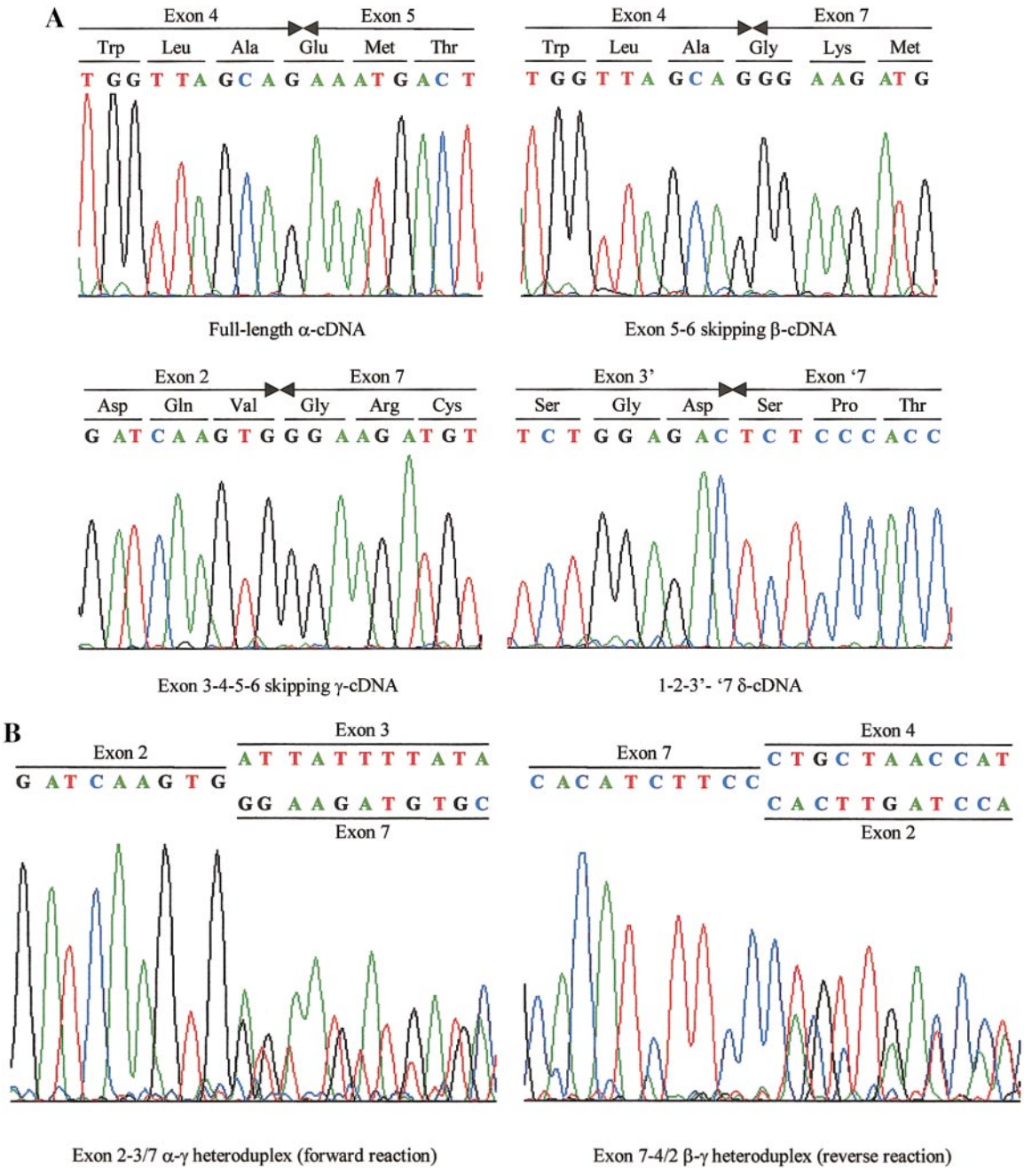

FIG. 3. (A) Schematic presentation of abnormally processed exon-skipping HCAR species in relation to the normal HCAR gene. (B) Sequencing chromatograms of the α-γ and β-γ heteroduplex formations.

RESULTS

Initially, an RT-PCR on total RNA, extracted from HeLa-cells, was performed. The primers HCAR-F and HCAR-R1 encompassed the entire coding region (51 bp upstream of the ATG start codon and 31 bp downstream of the TAG stop codon) and generated a full-length seven exon-encompassing HCAR-fragment of 1180 bp (here referred as the α-mRNA). After gel electrophoresis and ethidium bromide staining, an additional DNA fragment of 918 bp was observed. Sequencing of the 918-bp RT-PCR fragment revealed this to be an exon 5-6 skipping HCAR splice variant cDNA (β-mRNA).

To examine whether this aberrant splicing phenomenon was only restricted to the transformed HeLa cell line, a human multiple tissue cDNA panel (heart, brain, placenta, lung, liver, skeletal muscle, kidney,

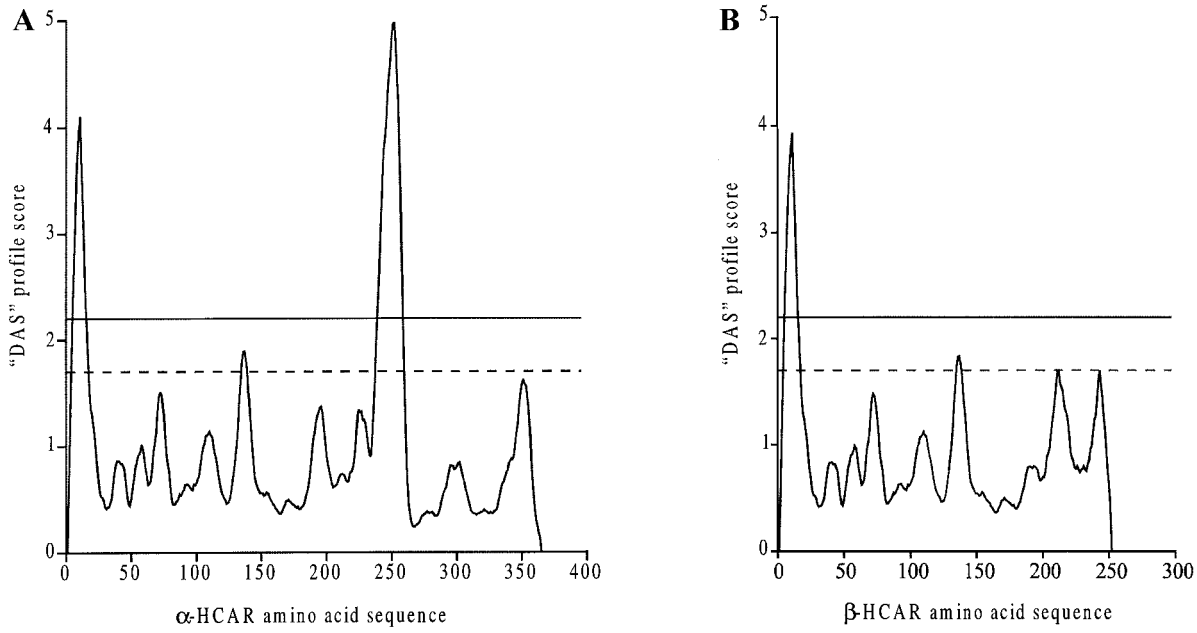

FIG. 4. Transmembrane segment prediction curves of the full-length HCAR α-protein (A) and the truncated β-variant (B). The dashed line represents a loose cutoff and the solid line a strict cutoff, with "DAS" profile scores of 1.7 and 2.2, respectively. Curve A shows a potential transmembrane segment from amino acid 238 to 258 (corresponding with the findings of Bergelson *et al.* (1) and Tomko *et al.* (2)). Curve B demonstrates the absence of a transmembranous region in the β-variant.

and pancreas) was subjected to semi-nested PCR. In the second PCR, the reverse primer was altered (HCAR-R2) resulting in a PCR generated amplicon with a reduced size of 10 bp. Gel electrophoresis after this second amplification showed the predominant seven exon-encompassing α-transcript (1170 bp) as well as the exon 5-6 skipping variant (β-transcript, 908 bp) to be present in all tissues except for skeletal muscle (Fig. 1). In heart, lung, and pancreas cDNA, an additional band of approximately 550 bp was amplified. Sequencing this fragment revealed a second variant as an exon 3-4-5-6 skipping transcript (547 bp, γ-variant). Several other bands of different sizes were visible on the gel, and to examine whether they were HCAR-specific, the semi-nested PCR-samples were blotted onto a nylon membrane and hybridized with digoxigenin-labeled HCAR-specific probes (Fig. 1). Two bands of medium intensity, a DNA-fragment around 600 bp in the placenta and an approximately 400-bp product in skeletal muscle were not visible on the Southern blot and sequencing analysis verified them to be aspecific nonHCAR PCR-fragments. A minor liver 400-bp product (δ) turned out to be HCAR-specific and consisted of exons 1-2, followed by the first 21 nucleotides of exon 3 and the last 97 nucleotides of exon 7. This δ-variant could only be revealed in the liver. Three splice variants (β, γ, and δ) were thus observed in human tissues, together with the full-length α-transcript (Figs. 2 and 3A). Other PCR-fragments visible on the gel as well as on the blot were subjected to sequencing analysis. Results showed that those fragments were heteroduplex formations between the alternative splice products and/or the normal alpha-transcript (Fig. 3B). An α-β, an α-γ, a β-γ, and a β-δ heteroduplex formation could be identified.

The control PCR with G3PDH-primers showed that approximately equal amounts of cDNA were provided in each cDNA preparation although there was a slight variation in band intensity (Fig. 1).

Results from the Transmembrane Prediction servers DAS (www.biokemi.su.se/~server/DAS/; 18) and PredictProtein (cubic.bioc.columbia.edu/predictprotein; 19) demonstrated that none of the three isoforms contained a potential transmembrane segment. In Fig. 4 the DAS transmembrane segment prediction curves of the β-variant as well as of the full-length α-protein are shown.

DISCUSSION

In addition to the originally identified HCAR α-form, we have identified three HCAR mRNA isoforms generated by alternative splicing of the HCAR pre-RNA. The β-variant is missing exons 5-6, the γ-variant is lacking exons 3-4-5-6, and the less common δ-variant is a 1-2-3′-′7 construct. All isoforms contain exon 1 and are therefore expressed from the same promoter. Several pseudogenes have been reported on chromosomes 15, 18, and 21 (2 loci), all exhibiting single base changes and small deletions and insertions (11). Sequencing analysis demonstrated that the splice variants reported here were 100% identical to the published func-

tional HCAR-sequence, indicating that all three isoforms result from a single preRNA transcribed from the functional HCAR-gene (GenBank Accession No. AF200465).

Two of the three splice variants (γ and δ) could only be detected after semi-nested PCR, suggesting a low amount of expression. This emphasizes again the extreme sensitivity of a semi-nested PCR but reveals also the inherent danger of overestimating the amounts and importance of the products amplified by these techniques. A reasonable explanation for the existence of the γ- and δ-variant could be that they result from splicing errors. In contrast, the β-variant could already be visualized after a single RT-PCR on HeLa total RNA. This would suggest that the β-isoform might exert an important function in the regulation of HCAR.

At the protein level, translation of the different mRNA isoforms would result in truncated proteins due to a frameshift downstream of the last preserved exon and consequently the occurrence of a premature stop codon. The β-isoform would give rise to a 252-amino acid protein with an altered amino acid sequence (difference of 62 aa) downstream of exon 4. Expression of the γ-variant theoretically results in the formation of an 89-amino acid protein with 19 different amino acids at the carboxy-terminal end, and an even shorter protein (81 aa) could be derived from the δ-variant with only the first 77 amino acids identical to the full-length protein amino-terminal end. *In vitro* transcription and translation assays (IVTT) should be performed to examine whether the different HCAR mRNAs encode stable proteins that are not degraded rapidly in the cell.

Functional assays by which HCAR-negative cells are transfected with cDNA encoding the truncated splice variants should also be carried out to determine their susceptibility to coxsackievirus and adenovirus entry. Wang and Bergelson (8) showed that HCAR cytoplasmic and transmembrane domains are not essential for viral attachment. Exons 6 and 7 constitute those latter domains and are therefore less important for viral interactions in contrast with exons 2-3 and exons 4-5 which comprise the Ig1 domain and Ig2 domain, respectively. The Ig1 domain alone was found to be sufficient for the adenovirus-binding activity whereas for coxsackievirus-binding both domains were thought to play a significant role (9, 13). Lacking exons 5-6 (β-variant) could theoretically result in a preserved adenovirus-binding capacity since the Ig1 domain and a major part of the Ig2 domain stay unaffected. Whether coxsackievirus-attachment would still be possible, remains to be elucidated. The γ and the δ variant proteins are crippled in both the Ig1 and Ig2 domains and are therefore unlikely to retain the virus-binding activity.

No potential transmembrane segments were found in any of the different splice variant proteins, which implied that if they would form stable proteins, they would be excreted as soluble forms in the intercellular compartment. This way, the β-variant could function as a soluble viral receptor that captures the virus and prevents it from entering the cell. Competitive inhibition of adenovirus infection *in vitro* by artificial HCAR soluble forms (cDNA constructs encoding a soluble HCAR protein, lacking the cytoplasmic tail, and the transmembrane domain) was previously reported by Roelvink *et al.* (3) and Van 't Hof and Crystal (17), with levels of inhibition of 95 and 60%, respectively. The presence of soluble receptors *in vivo* could thus have a significant impact on the outcome of adenovirus-based gene therapy. Successful gene delivery requires viral entry into the target cell via specific receptor-mediated uptake and tissue HCAR levels are positively correlated with adenoviral infectivity (15, 17, 20, 21). The presence of soluble HCAR isoforms could prevent viral uptake and thereby decrease the efficacy of adenovirus-mediated gene transfer. Tissue- and species-specific differential CAR-mRNA splicing has been suggested previously. When hybridizing a human poly-A^+ multiple tissue Northern blot with a HCAR-specific probe, Fechner and colleagues (6) detected mRNAs of different sizes ranging from 7 to 2.2 kb. Similar results were obtained by Tomko *et al.* (2). The nature of these different mRNA species has however still to be clarified. In addition, Fechner *et al.* (6) discovered the existence of two different CAR homologues (referred as CAR1 and CAR2) in rat and in man which had also been described for mouse by Bergelson *et al.* (22). There was no difference between the amino acid sequences of HCAR1 and HCAR2 up to amino acid position 339 but a divergent and 13-amino-acid shorter C-terminus was identified in HCAR2. This is due to the existence of an alternative exon 27 kb downstream of exon 7 (12). Both homologues function equivalently in group B coxsackievirus and adenovirus attachment.

We describe, for the first time, the existence of three aberrant splice variants of the HCAR-gene. Further investigation is needed to clarify their functional significance both on group B coxsackie- and adenovirus binding and on the normal cellular function of HCAR.

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