Identification and tissue distribution of novel KET/p63 splice variants

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Abstract The human p53 protein family comprises three members – p53, p63 and p73. Whereas only one p53 variant is known multiple isoforms of p63 and p73 have been described. Depending on the isoform p63 influences p53-responsive genes in a p53-like or -distinct manner. We have cloned multiple splice variants of keratinocyte transcription factor (KET), the rat ortholog of human p63. Several tissue specific variations of exon 1 resulting in different amino-terminal ends were identified. Transactivation properties of the splice variants inversely correlated with the length of the N-termini as determined by activation of the p53-responsive p21 promotor. Multiple KET isoforms are colocalized in different rat tissues. The amino-terminal truncated form ΔNKETα is expressed in epithelial tissues, while expression of the most p53-like KET isotype TAKETγ was detected in skeletal muscle. Expression of a major KET variant appears to be a cell-type specific rather than a differentiation specific phenomenon.

Key words: Splice variants;
Keratinocyte transcription factor; Transactivation

1. Introduction

The tumor suppressor p53 fulfills a key function concerning the regulation of cell cycle and apoptosis [1]. Two new proteins named p63/KET and p73 have been described which are up to 75% identical to p53 in its evolutionary conserved domains [2–4]. Several features of the transcription factor p53, namely the amino-terminal transactivation domain (TA), the core DNA binding domain (DBD) and the carboxy-terminal oligomerization domain (OD) are sufficiently conserved in p63 and p73 to bind to p53-responsive elements and to possibly exert a p53-like effect on gene transcription [5]. In addition a sterile alpha motif (SAM)-like sequence found at the carboxy-terminus of p63 and p73 is thought to mediate protein–protein interactions [6,7]. Transcription assays have indicated that this p63 carboxy-terminus may exert an inhibitory effect on the p53-like activity of p63 [8].

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Abbreviations: KET, keratinocyte transcription factor; SAM, sterile alpha motif; TA, transactivation domain; ΔN, amino-terminal truncated; HGPRT, hypoxanthine guanine phosphoribosyl transferase; RPA, ribonucleic acid protection assay; GAPDH, glyceraldehyde 3 phosphate dehydrogenase; ID, identifier element; PCR, polymerase chain reaction; RT, reverse transcription; RACE, rapid amplification of cDNA ends

In contrast to the single known p53 protein p63 exists in mammals in six major isotypes, including two variants at the amino- and three at the carboxy-terminus [9]. An alternative promotor in intron 3 results in the transcription of amino-terminal truncated (ΔN) p63 mRNAs lacking the amino-terminal TA and therefore the ability to mediate p53-like transactivation [8].

Alternative splicing of the p63 mRNA at the 3′-end leads to at least three different isoforms. In addition to the full length p63α, deletion of exon 13 during splicing results in p63β proteins which have lost a part of the carboxy-terminal SAM domain [10]. Alternatively to exon 11, exon 15 can be added to the 3′-end of exon 10. This shortest p63 isoform termed p63γ is most similar to the carboxy-terminal end of p53.

Due to different transactivation activities of the p63 isotypes [8] and because of the high number of possible, functionally diverse p63 forms it is important to understand the expression pattern of the splice variants. In the following study additional splice variants of rat keratinocyte transcription factor (KET) and human p63 were identified, the transactivation properties were determined and their tissue distribution was characterized.

2. Materials and methods

2.1. RNA preparation

For RNA isolation tissues were dissected from adult Wistar rats and stored at −70°C. The tongue epithelium was prepared directly after dissection from the tip of rat tongues by collagenase/dispase treatment [11]. Human tissue samples were removed at autopsy and total RNA was isolated from all tissues using RNAclean (Hybaid-AGS, Heidelberg, Germany) according to the manufacturer's instructions.

2.2. Rapid amplification of 5′-cDNA ends (5′-RACE)

Novel 5′-ends of the KET mRNA were identified with the 5′/3′-RACE kit (Roche, Mannheim, Germany) according to the manufacturer's description. cDNA synthesis was carried out with rat tongue total RNA and rat placenta total RNA and the gene specific oligonucleotide KET TA rev (Table 1). The oligonucleotide KET TA3 rev and the supplied anchor primer were added to the anchor polymerase chain reaction (PCR). A second round of PCR was performed with the oligonucleotide 5′-cag aaa atc cca gat atg ctg g-3′ and supplemented nested primer. PCR products were subcloned and sequenced.

2.3. Ribonuclease protection assay (RPA) and reverse transcription (RT)-PCR analysis

For RPA, contaminating DNA in the rat tissue total RNA preparation was removed by DNase I digestion (1 U per 20 μg RNA, 15 min, 37°C in 1×transcription buffer, Roche). A total of 20 μg of RNA per assay was incubated with 5×10^5 cpm [α-^{32}P]UTP (Amersham, Freiburg, Germany) labeled KET specific probe at 42°C overnight in hybridization buffer (RPA-III-Kit, Ambion, Austin, TX, USA). A glyceraldehyde 3 phosphate dehydrogenase (GAPDH) specific probe (5×10^4 cpm) was included as internal control. Riboprobes were generated by in vitro transcription of 1 μg linearized plasmid

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DNA with T3 or T7 RNA polymerases (Roche) or SP6 RNA polymerase (Stratagene, La Jolla, CA, USA). The KETcore-p probe of 370 base pairs (bp) length was used to hybridize to a 289 bp long KET mRNA fragment (nucleotides (nt) 1017–1317, GenBank accession number (GB): Y10258). Expression of TA was analyzed with TA2KET-p (333 bp) which included a 303 bp long KET TA2 sequence (nt 148–450, GB: Y10258). This probe allowed simultaneous detection of the KET TA1 or KET TA3 splice variant as a 241 bp long protected cRNA fragment common to all KET TA splice variants. The KET ΔN splice variant was identified with the ΔNKET-p probe (250 bp) which detected a 170 bp long sequence (nt 1–169, GB: AJ277447). The 300 bp long KETα-p probe protects a 280 bp fragment of KETα (nt 3208–3487, GB: AJ277447). A 254 bp long KETγ fragment (nt 1202–1433, GB: AJ277450) was protected by the 310 bp long transcript named KETγ-p. GAPDH-p (217 bp) hybridized to 200 bp of the GAPDH mRNA (nt 351–550, GB: X02231). RNase treatment was carried out with RNase A/T_1 (RPA-III-Kit, Ambion) in a 1:100 dilution in RNase digestion buffer (30 min, 37°C). After separation of cRNA fragments in a 8% polyacrylamide gel radioactive bands were analyzed on the dried gel with the phosphoimager system Fuji BAS-1800II reader and the software package AIDA (Raytest, Straubenhardt, Germany).

For RT-PCR 2 μg of total RNA was reverse transcribed using Superscript II reverse transcriptase (BRL Life Technologies, Eggenstein, Germany) and random hexamer primers (Amersham). Splice variant specific PCR was carried out with Taq PCR Master Mix (Qiagen, Hilden, Germany) under the following conditions. 1/20 of the cDNA was used for PCR amplification with 35 cycles (94°C for 30 s, 55°C for 30 s, 72°C for 1 min). The temperature program was modified for KET TA1 (58°C for 30 s) and KET TA3 (72°C for 1 min and 12 s). Specific KET cDNA sequences were amplified with the forward and reverse oligonucleotides listed in Table 1. A second PCR approach with a KET 5′-end forward and 3′-end reverse oligonucleotide identified the expressed KET 5′/3′-end combination. The cDNAs TAKETα, TAKETγ, ΔNKETα and ΔNKETγ were amplified with the ELONGASE Enzyme Mix (BRL Life Technologies) in a PCR as described above with a modified elongation step (68°C for 2 min and 30 s). PCR fragments were visualized with ethidium bromide after separation of equal volumes of each reaction on a 1.2% (w/v) agarose gel.

2.4. Transcriptional activation assays

For heterologous expression of p63, PCR products of the TA1p63γ, TA2p63γ, TA4p63γ and TA2p63α coding sequences were cloned in the pcDNA3.1/Myc-His A vector (Invitrogen, Groningen, The Netherlands). The rat KET TA3 cDNA sequence was added to the 5′-end of the human TA4p63γ leading to the expression of chimeric proteins with a rat KET TA3 N-terminus, a human p63 core domain and a human p63γ C-terminus. The pcDNA3/p53wt (wild type) plasmid was a gift of Manfred Westphal (UKE, Hamburg, Germany).

Cotransfection of Saos-2 cells with the p63 expression construct, the p53 reporter plasmid pG13/p21/luciferase (kindly provided by Wolfgang Deppert, HPI, Hamburg, Germany) and the pcDNA3.1/Myc-His/*lacZ* vector (Invitrogen) was performed with Effectene (Qiagen). After subsequent cultivation for 24 h luciferase activity was quantified by chemiluminescence signal detection with the luciferase assay kit (Promega, Madison, WI, USA) and the MicroLuminat LB 96 P luminometer (EG and G Berthold, Bad-Wildbad, Germany). Transfection efficiency was evaluated by cotransfection of the pcDNA3.1/Myc-His/*lacZ* plasmid and determination of the β-galactosidase activity with the Galacto-Light Chemiluminescent Reporter Gene Assay (Applied Biosystems, Bedford, MA, USA). Triplicate experiments were performed.

3. Results

3.1. Structure of the KET splice variants

Starting with known sequences of human p63 cDNA clones [12] and the rat KET cDNA [3] new rat KET and human p63 splice variants with altered open reading frames were identified. The 5′-RACE technique was used to elucidate the number of possible different 5′-ends of KET and human p63 mRNAs. For clarification, all KET/p63 isoforms including the transactivation domain encoded by exons 2 and 3 are named KET/p63 TA. Upstream of exon 2 exist at least three different KET exons, 1, 1′ and 1″, which give rise to the N-termini KET TA1, TA2 and TA3, respectively (Fig. 1A). Exons 1 and 1′ are also found in human p63. The protein-encoding sequences of exons 1 and 1′ are 97% identical between rat and human while 74% identity is observed in the 5′-UTRs. Analysis of 5′-RACE products led in all cases to the identification of a putative start methionine with a preceding stop codon in at least one species. Exon 1 encodes four amino acids in addition to KET/p63 TA. The exon 1′ nucleotide sequence of KET/p63 TA2 determines 21 amino acids and was described earlier in man, rat and mouse as TA*p63 [2,3,13]. The protein sequences of KET/p63 TA1 and TA2 are 100% identical in the species studied.

A second splice site 8 bp downstream of the intron 1 splice acceptor site was found in 5′-RACE PCR products of human skin total RNA (Fig. 1A, arrow).

5′-RACE PCR products and subsequent RT-PCR experiments indicated the existence of another 5′-end for the KET mRNA in placenta termed KET TA3. KET TA3 PCR products of different length were identified including and lacking a repetitive element between exons 1″ and 2. This insertion of 36 bp shows high identity to rat identifier element (ID) sequences [14]. Exon 1″ appears to be specific for the rat because it was not detected in human placenta total RNA.

KET mRNAs lacking the transactivation domain are transcribed from an alternative promotor in intron 3. These mRNAs begin with exon 3′ and encode the amino-terminus KET ΔN (Fig. 1B). The putative 5′-UTRs of the rat and human exon 3′ identified by screening of a cDNA library differed in length by 2 bp and are 81% identical. One amino

Table 1
PCR primers and products for KET

Identification	Forward primer	Reverse primer	Size (bp)	Position (nt)	KET GB
KET TA	5′-gcc cat tga ctt gaa ctt tgt g-3′	5′-ctg gaa gga cac atc gaa gct g-3′	338	356–693	Y10258
KET TA1	5′-ggc cac tct atg tca agg gct cta a-3′	5′-gag ccc cag gtt cgt gta ctg tgg-3′	357	210–566	AJ277446
KET TA2	5′-tct gat ggc att tga ccc tat tg-3′	5′-gag ccc cag gtt cgt gta ctg tgg-3′	470	26–495	Y10258
KET TA3	5′-gtg gga taa tgg caa aag gca g-3′	5′-tga gtc ttg cat gcg gat ac-3′	297, 259	–	–
KET ΔN	5′-gga agc aga gaa gag gag agc-3′	5′-ctg gaa gga cac atc gaa gct g-3′	303	74–376	AJ277447
KETγ	5′-ctt cag gaa tga gct tgt gg-3′	5′-gtg gat gtg tgc ctt tga gc-3′	253	1512-(1764)	AJ277449
KETα and β	5′-tgg cat ggg agc caa cat tc-3′	5′-tcc agg atc ccc tcc cag at-3′	318, 224	1638–1955	Y10258
TAKETγ	5′-gcc cat tga ctt gaa ctt tgt g-3′	5′-gtg gat gtg tgc ctt tga gc-3′	1388	356–1723	AJ277449
ΔNKETα	5′-gga agc aga gaa gag gag agc a-3′	5′-aaa gaa atg gtc tgg cgg ag-3′	1719	74–1792	AJ277447
HGPRT	5′-cgt cgt gat tag tga tga tga-3′	5′-ttc aaa tcc aac aaa gtc tgg-3	526	119–644	S79292

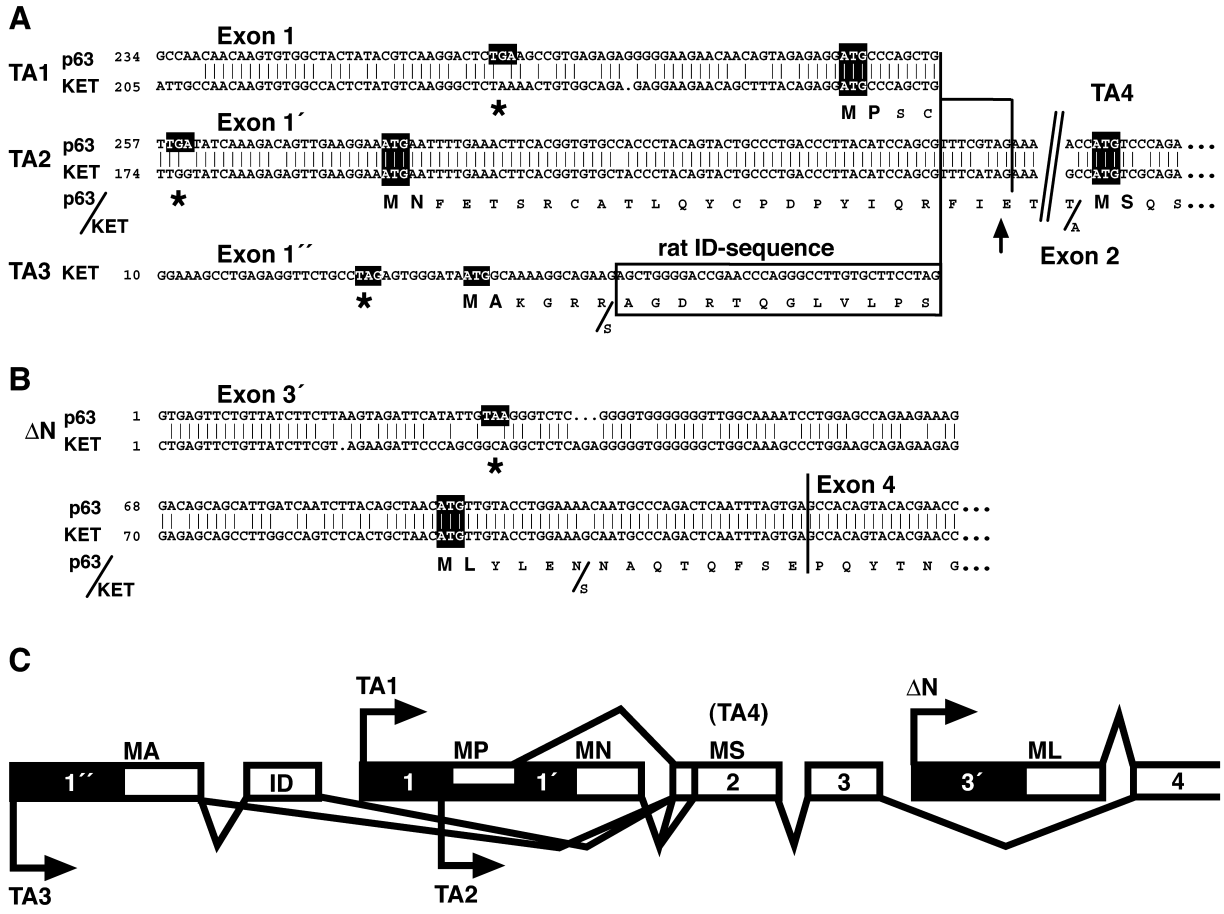

Fig. 1. A and B: Nucleotide sequences and deduced amino acid sequences of rat KET in comparison to the human p63 nucleotide sequences. The alignment indicates the high identity of the 5′-exons between the two species. The asterisks mark the in-frame stop codons. Amino acids are abbreviated in single letter code. Start and stop coding nucleotides are shown in white on a black background. The rat ID sequence in the KET TA3 5′-end is enclosed in a box. Bold vertical bars separate two exons from each other while small vertical bars mark identical nucleotides between the orthologs KET and p63. The arrow points to the alternative splice acceptor site of exon 2. //: Nucleotides of exon 2 omitted. C: Possible transcription and translation starts for KET are shown in the putative exon/intron structure of the KET gene 5′-region. Open white boxes highlight translated mRNA sequences while closed black boxes indicate 5′-UTR regions. Arrows indicate possible transcription start sites while putative translation start methionines and the respective second amino acids are written in capital letters. The positions of exon 1″ and ID are hypothetical.

acid exchange is observed in the protein coding region of exon 3′.

The complex splicing events of the KET 5′-end are summarized in Fig. 1C. In general, each long open reading frame begins in the respective first exon starting with the two amino acids MA, MP, MN or ML. However, when any of the first exons is added to the alternative splice acceptor in exon 2, the resulting frame shift would allow translational start of KET only in exon 2 beginning with the two amino acids MS. This isoform is termed KET/p63 TA4 and corresponds to TAp63 in Yang et al. [2]. The depicted organization of exons 1 and 1′ was derived from the analysis and comparison of 5′-RACE clones with the human genomic DNA sequences on BAC clone 71B1 (Research Genetics, St. Louis, MO, USA). Protein coding sequences for TA1 and TA2 are located in close vicinity separated by 240 nucleotides. Both coding sequences are followed by conserved GU splice donor sites. At present it can not be decided whether exons 1 and 1′ arise from a common primary transcript by alternative usage of the GU splice donor or whether they constitute independent transcripts.

Additional splice variants of the KET mRNA were identified (Fig. 2A) in the central part of the molecule. An alternative splice donor at the 3′-end of exon 8 removes 12 bases from the KET mRNA between the regions encoding the DNA binding and the tetramerization domain. This truncated exon 8, named 8′, is conserved in mRNAs of man, rat and mouse. KET mRNAs with exon 8′ do not encode the four amino acids GTKR comprising a putative protein kinase C phosphorylation site.

In analogy to p63 extensive splicing is observed at the 3′-end of the KET mRNA. The KETα mRNA comprises exons 11–14. Removal of exon 13 in the KETβ mRNA leads to the

Table 2
GenBank accession numbers for the identified KET splice variants

KET splice variant	GB accession number
TA1KETα	AJ277446
TA2KETα	Y10258
ΔNKETα	AJ277447
TA1KETγ	AJ277448
TA2KETγ	AJ277449
ΔNKETγ	AJ277450
TA2KETβ	AJ277451
TA1KETβ	AJ277452
ΔNKETβ	AJ277453

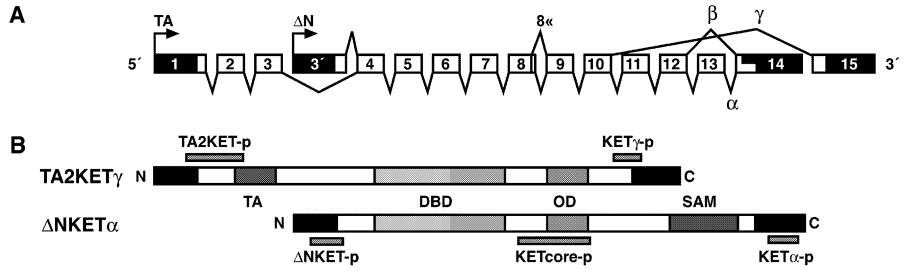

Fig. 2. A: The scheme illustrates the exon/intron structure of the KET gene. The alternative transcript starts are designated TA and ΔN. An alternative splicing event results in a truncated exon 8 named exon 8′. Different 3′-ends of the KET mRNA are described as α, β and γ in analogy to p63. B: Two KET mRNAs coding for the isoforms TA2KETγ and ΔNKETα are shown to indicate the positions of different hybridization probes (-p) used in the RPA. The names refer to their splice variant specificity. Boxes in different shadings highlight known KET domains.

loss of a functional SAM domain. Five additional amino acid codons are followed by a stop codon in the new reading frame of exon 14.

KETγ is a consequence of exon 15 being alternatively spliced to the 3′-end of exon 10. The nucleotide sequences of the described splice variants are deposited at GenBank (Table 2).

3.2. KET expression in different rat tissues

KET mRNA expression in rat tissues was investigated with splice variant specific probes by RPA (Fig. 2B). KET mRNAs beginning with exon 1′ or the KET exon 3′ were detected with the RPA probes TA2KET-p and ΔNKET-p, respectively. KETcore-p binds to all KET mRNAs irrespective of their 5′-end or 3′-end. In order to discriminate between the different 3′-ends of KETγ and KETα, the probes KETγ-p and KETα-p were used.

KET expression was detected in tongue epithelium, skin, placenta, thymus, muscle and prostate. Heart was the only tissue studied without detectable levels of KET expression (not shown).

RNAs beginning with exon 1′ (KET TA2) were found in skin, thymus and muscle (Fig. 3A, RPA, 303 bp fragment). The probe TA2KET-p allowed detection of all TA variants irrespective of the specific first exon because the entire exon 2 sequence was included. Protected cRNA fragments of 233 bp and 241 bp length in skin, placenta, thymus and muscle pointed to the existence of other or alternatively spliced sequences. While the 241 bp fragment represents the entire exon 2, the 233 bp long cRNA fragment may be explained by the usage of the second splice acceptor site in exon 2.

The presence of the TA region encoded by exons 2 and 3 in all tissues studied except prostate was demonstrated by RT-PCR (Fig. 3A, KET TA). Exon 1 specific RT-PCR experiments showed that KET TA1 and KET TA2 are coexpressed in skin, thymus and muscle. Although RPA and RT-PCR results suggested KET TA mRNA expression in rat placenta, neither KET TA1 nor KET TA2 5′-ends were detected in this tissue. PCR amplification of KET TA3 proved that this variant represented the major TA product of placenta. The two bands of 259 bp and 297 bp are amplicons with or without the rat ID sequence. Our preparation of the rat placenta does not exclude KET TA3 expression in fetal tissues as part of the placenta.

KET ΔN sequences starting with exon 3′ were found in tongue epithelium, skin, placenta, thymus and prostate when examined with RPA and RT-PCR (Fig. 3B). The KET ΔN splice variant was not detected in skeletal muscle despite successful amplification of the hypoxanthine guanine phosphoribosyl transferase (HGPRT) control.

As predicted by the results described above, KETcore was detected in all tissues depicted in Fig. 4A. The RPA revealed no tissue specific difference in exon 8 versus 8′ expression. Significant KETα expression was identified in all investigated

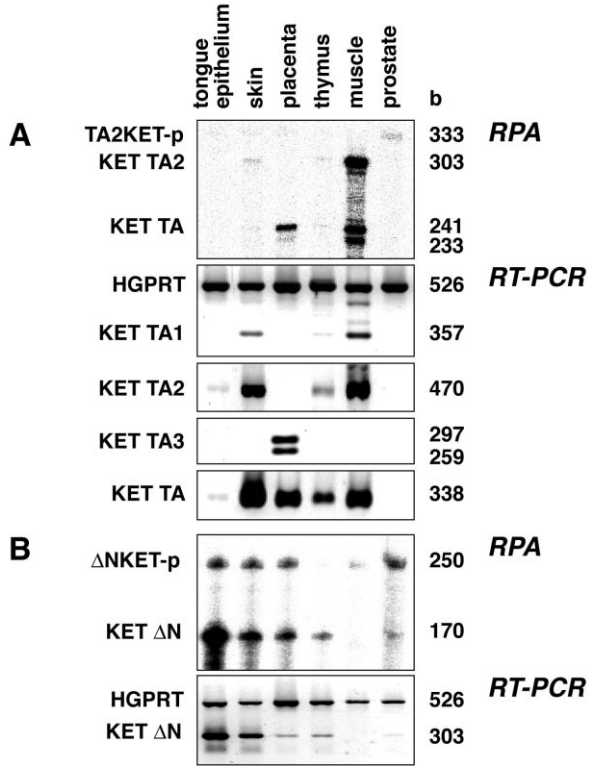

Fig. 3. KET TA and KET ΔN expressions were analyzed in different rat tissues using RPA and RT-PCR. The positions of the full-length RPA probes remaining after digestion are indicated by -p. A: The tissue distribution of different 5′-ends of the KET mRNA including the transactivation domain was analyzed. RPA detected the specific expression of KET TA2 (303 bp) and as smaller cRNA fragments the expression of KET TA1/KET TA3 (241 bp and 233 bp). The RT-PCR experiments discriminated between KET TA1, TA2 and TA3 expressions. A further RT-PCR experiment shows the amplification of all KET TA sequences containing the transactivation domain in exon 2 independent of the first exon. B: KET mRNAs resulting from the alternative promotor were detected by RPA and RT-PCR. Amplification of a HGPRT fragment was used as positive control.

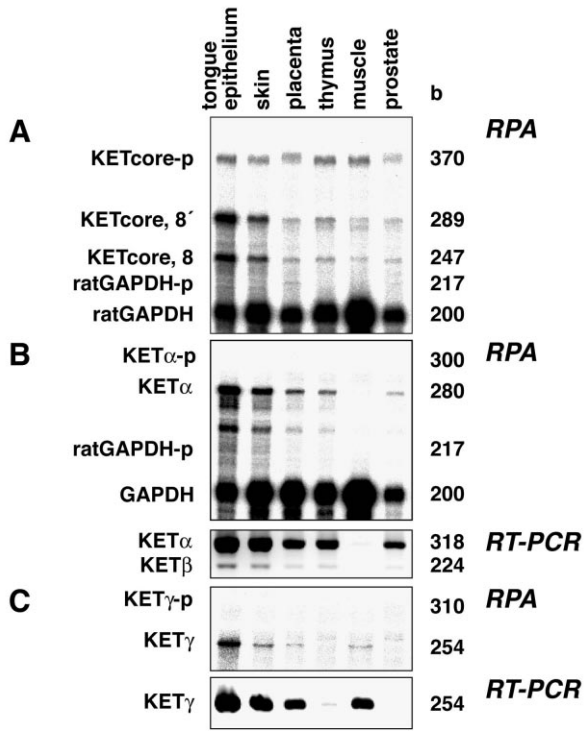

Fig. 4. RPA and RT-PCR analyses localized KET expression in different rat tissues. A: Detection of KET regardless of the 5′-end and the 3′-end. KETcore-p allowed the simultaneous detection of exon 8 (247 bp) and 8′ (289 bp). Detection of GAPDH monitored the amount of total RNA in the assay. B: The tissue distribution of KETα and KETβ was analyzed with RPA and RT-PCR. Co-detection of the GAPDH mRNA was used as a positive control. C: RPA as well as RT-PCR show the expression profiling of the alternative exon 15, KETγ.

tissues except muscle which is barely positive in RT-PCR experiments (Fig. 4B). KETα specific RT-PCR allowed parallel detection of the KETβ deletion splice variant. The RT-PCR results suggest a fixed ratio of about 10:1 for KETα to KETβ. RPA demonstrated KETγ expression in tongue epithelium, skin, placenta and muscle but not in prostate (Fig. 4C). The result was confirmed by RT-PCR. In thymus very weak expression of KETγ was detectable.

The above described RPA and RT-PCR experiments showed the tissue distribution of individual splice and transcription modules. In order to directly demonstrate the combination of the major amino- and carboxy-termini of KET, specific primers were used to amplify the entire coding region.

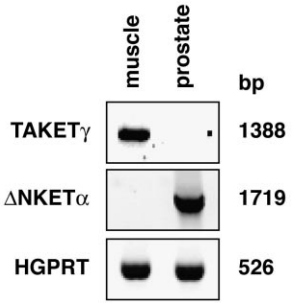

Fig. 5. RT-PCR analysis identified the combination of KET splice variants in skeletal muscle and prostate of rat. A HGPRT cDNA fragment was amplified as positive control.

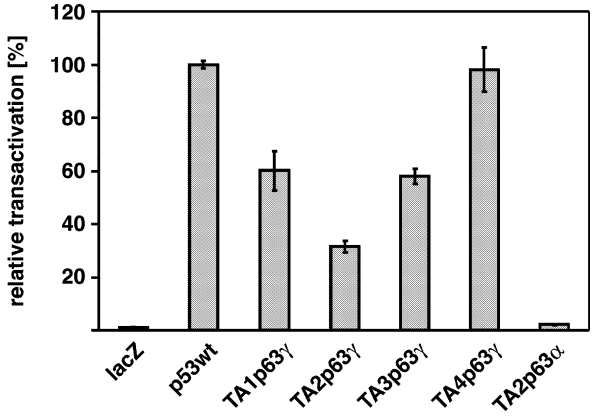

Fig. 6. Transactivation activity of different p63 N-termini was analyzed in Saos-2 cells cotransfected with the pG13/p21/luciferase reporter plasmid. Quantification of β-galactosidase expressed from a second cotransfected plasmid was used to normalize for transfection efficiency in all experiments. Transactivation is shown relative to the p53wt activity (100%). Error bars: ±S.D.

In skeletal muscle, expression of KET was restricted to the TAKETγ isoform, while in prostate the ΔNKETα form was predominant (Fig. 5). All other tissues displayed various isoforms. ΔNKETα as well as TAKETγ were detected in thymus total RNA. Three splice variants TAKETα, ΔNKETα and ΔNKETγ are present in tongue epithelium. All four splice variants TAKETα, TAKETγ, ΔNKETα and ΔNKETγ are expressed in skin and placenta (results not shown).

3.3. Transactivation activity of different KET/p63 N-termini

In order to detect possible differences in the transactivation potentials of the newly identified KET/p63 5′-end splice variants, we employed the ability of the p63 DNA binding domain to bind to p53-responsive elements in the human p21 gene. The level of transcriptional activation of a luciferase reporter plasmid containing 2.4 kb promotor sequence of the human p21 gene was analyzed by transient transfection of p53 negative Saos-2 cells (Fig. 6). TA1p63γ and the chimeric construct TA3p63γ, consisting of the rat KET TA3 N-terminus, the human core and the human p63γ C-terminus, transactivate luciferase transcription to about 60% when compared to p53wt. TA2p63γ shows only 40% transactivation activity while TA2p63α exhibits almost no transactivational potential possibly due to the autoinhibitory p63α C-terminus. The transactivation rate of TA4p63γ, the N-terminus of which is most similar to that of p53, does not differ from activity mediated by p53wt itself [2].

4. Discussion

The variation of the 5′-end of the KET mRNA is remarkable and raises the question for the translational start methionine that is really used. The first methionine in exon 2 corresponds to the start methionine in p53 and was suggested as amino-terminus for p63 TA and p51 [2,15]. It is evident that the possible KET protein diversity would be drastically reduced if all start methionines in the first exons including KET TA3 were ignored by the translation apparatus. However, protein sequences at the extreme amino-terminus of p63 have an influence on the transcriptional activity of the protein. The isoform with the shortest N-terminus, p63 TA4, showed

the highest transactivation activity followed by p63 TA1 with additional 22 amino acids and p63 TA2 with 39 more amino acids as indicated by our results. Transcription activity might be influenced by the length of the N-terminus of different p63 TA isoforms. Thus it is conceivable that biological activity of KET/p63 is not only determined by the choice between TA and ΔN isoforms but in addition fine tuned by the use of different TA variants. The nearly perfect conservation of amino acids at the extreme amino-terminus in all species studied strongly supports a possible functional role.

Expression of a major KET isotype seems to be a cell-type specific phenomenon. This was demonstrated by the predominant detection of the KET isoform ΔNKETα in prostate and TAKETγ in skeletal muscle. In complex tissues the relative amounts of cell types expressing different KET isotypes will determine the observable overall expression pattern of KET variants. An example comes from the analysis of KET expression in tongue epithelium and skin. Enzymatic preparation of the tongue epidermis reduces the amount of muscle cells. This is reflected by a significant reduction of KET TA expression in the tongue epithelium in comparison to skin. While the strong expression of ΔNKETα in keratinizing epithelia was reported earlier [2], detection of TAKETγ expression in skeletal muscle is a new observation. The existence of this splice variant, which is most similar to p53, may suggest a possible role of KET/p63 in terminal differentiation of skeletal muscle cells.

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