Reverse transcription-polymerase chain reaction products of alternatively spliced mRNAs form DNA heteroduplexes and heteroduplex complexes

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Reverse transcription-polymerase chain reaction (RT-PCR) is frequently used to simultaneously detect mRNA isoforms, which are generated by alternative splicing. Here we characterize two previously unrecognized RT-PCR products of vascular endothelial growth factor (VEGF) RNA. DNA products with apparent sizes of 600 and 1200 base pairs (bp) were detected at high cycle numbers. Heat denaturation of the smaller product and subsequent reannealing revealed that it was a heteroduplex consisting of two different DNA strands. These were identified by DNA sequencing as the amplification products of two VEGF transcripts, i.e. VEGF121 and VEGF165, which differ by the presence of one exon. S1 nuclease analysis showed that this exon is bulged out as a single-stranded loop. Purified heteroduplexes in solution were found to form a 1200-bp DNA product which could be reconverted into 600-bp DNA heteroduplexes by mild denaturation at 70 °C. These findings suggest that this product is formed by base pairing of complementary heteroduplex loops and represents a novel four-stranded DNA structure.

Alternative splicing generates different mRNA species from a common precursor molecule, and these mRNAs may encode proteins that differ in their biochemical and biological properties. One example for this phenomenon is vascular endothelial growth factor (VEGF) which is an important regulator of angiogenesis and blood vessel permeability (1, 2). Of its three transcripts, only VEGF165 and VEGF189 bind heparin, which differ by the presence of one exon. S1 nuclease analysis showed that this exon is bulged out as a single-stranded loop. Purified heteroduplexes in solution were found to form a 1200-bp DNA product which could be reconverted into 600-bp DNA heteroduplexes by mild denaturation at 70 °C. These findings suggest that this product is formed by base pairing of complementary heteroduplex loops and represents a novel four-stranded DNA structure.

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MATERIALS AND METHODS

RT-PCR—Total RNA was extracted from the human epidermoid cell line A431 with RNazol (Cinna/MRC, Cincinnati, Ohio) and reverse-transcribed as described previously (2). For amplification of VEGF-specific cDNAs, a sense primer annealing within exon 1 (5'-CCA TGA ACT TTC TGC TGT CTT-3') and an antisense primer derived from the untranslated region of exon 8 (5'-TCG ATC TTC TCT TCA CTT-3') (2) were used in a reaction involving denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 1 min. The number of cycles was varied from 25 to 40. PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide.

DNA Preparation from Agarose Gels—DNA was prepared from bands of tris borate-EDTA-agarose gels with the Qiaex II gel extraction kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. DNA was eluted from Qiaex particles with water at a temperature of 50 °C for 15 min.

S1 Analysis—50 ng of DNA were incubated with 20 units of S1 nuclease (Boehringer Mannheim, Germany) in S1 buffer (33 mM Na-acetate, 50 mM NaCl, 0.03 mM ZnSO4·7H2O, pH 4.5) for 1 h at 37 °C. Reaction products were separated electrophoretically on a 2% agarose gel and blotted onto a nylon membrane. For Southern hybridization, the oligonucleotides used as PCR primers were 32P-labeled with terminal transferase (Stratagene, Heidelberg, Germany) and incubated with the blot at 65 °C for 2 h. Membranes were washed and exposed to x-ray film (Kodak, Rochester, NY).

RESULTS

Detection of aberrant RT-PCR products—To detect VEGF splice variants expressed at low levels, PCR was performed on cDNA from A431 cells at high sensitivity conditions. In addition to the previously described isoforms VEGF121, VEGF165, and VEGF189, (2) which yield amplification products of 526, 658, and 730 bp, respectively (Fig. 1A), an additional band appeared when the number of PCR cycles was raised over 30 (Fig. 1B). This DNA band had an apparent size of approximately 600 bp on a 1.5% agarose gel. Compared with the other bands, its abundance increased overproportionally during the last PCR cycles (Fig. 1B). These kinetics suggested that the mechanism of its formation was different from the one that generates standard PCR products. Southern hybridization with an oligonucleotide recognizing all known splice forms of VEGF confirmed that the 600-bp band originated from VEGF
cDNA (data not shown). An additional band of the apparent size of 1200 bp was found when we tried to maximize the amplification yield by combinations of high cycle number, high amounts of cDNA template, and high polymerase concentrations (see below).

Identification of the Components of the Aberrant RT-PCR Product—For further analysis, the 600-bp band was purified from an agarose gel as described under “Materials and Methods.” Aliquots of a common reaction mixture were distributed to a series of tubes that were removed from the thermocycler after 25, 28, 31, 34, 37, and 40 PCR cycles (lanes 1 to 6, respectively). Equal volumes were loaded on a 1.5% agarose gel containing ethidium bromide. The position of the aberrant PCR product is indicated by an arrow. Sizes of a DNA length marker (M) are shown in base pairs.

S1 Nuclease Analysis of the VEGF<sub>121</sub>/VEGF<sub>165</sub> DNA Heteroduplex—To elucidate the structure of the VEGF<sub>121</sub>/VEGF<sub>165</sub> heteroduplex, DNA from the 600-bp band was digested with S1 nuclease and electrophoresed in parallel with an undigested aliquot (lanes 1 and 2), VEGF<sub>121</sub> PCR product (lanes 3). Blots were hybridized either with radioactively labeled 5′ PCR primer oligonucleotide (panel A) or with the 3′ PCR primer oligonucleotide (panel B). The sizes of the bands as determined by comparison with a DNA length standard are shown on the left side. Heteroduplex-derived bands specifically hybridizing with one of the two oligonucleotides are indicated by arrows. C, schematic representation of the VEGF PCR products involved in heteroduplex formation and of the two types of heteroduplexes. DNA strands are represented by lines with arrowheads at their 3′ end to indicate their orientation. The segment of VEGF<sub>165</sub> corresponding to exon 7 is shown as a bold line. Interactions between heteroduplexes that mediate the formation of a heteroduplex-duplex are indicated by double-headed arrows.

S1 cleavage site at the position of the VEGF<sub>165</sub>-specific exon 7, it became evident that this region is single-stranded in the complex. Both the 100- and the 420-bp band are generated by cleavage of both heteroduplex DNA strands at the site of sequence divergence. Because of incomplete digestion, an intermediate product, in which only the exposed single-stranded DNA becomes detectable when the 3′ strand of the complex is cleaved, was present in the resulting reaction mixture. Both probes hybridized to that intermediate of 520 bp length (Fig. 3, A and B). These results suggest that the VEGF<sub>165</sub>-derived heteroduplex DNA strand adopts an Ω-like conformation in which the sequence corresponding to exon 7 forms a single-stranded loop (Fig. 3C).

Because either the sense or antisense strand of both VEGF<sub>121</sub> and VEGF<sub>165</sub> PCR products can participate in heteroduplex formation, two types of complexes appear. In one of them, the VEGF<sub>165</sub>-derived exon 7 sequence is exposed in sense
orientation. In the other heteroduplex-type, the single-stranded loop is in antisense orientation (Fig. 3C). The sequence complementarity in the loops suggests that two heteroduplexes might interact through base pairing in that region (as depicted by double arrows in Fig. 3C).

Identification of the Second RT-PCR By-product as a Complex of Heteroduplexes—The second aberrant PCR product (1200-bp band), which was detected after maximized DNA amplification (Fig. 4A), was assumed as a potential complex of two VEGF$_{121}$/VEGF$_{165}$ heteroduplexes because it was exactly twice as large. To investigate whether this product represented a complex stabilized by weak interactions such as local base pairing, PCR products were subjected to mild heat denaturation (heating to 70 °C for 10 min and subsequent chilling on ice). Whereas perfectly double-stranded DNA species and the VEGF$_{121}$/VEGF$_{165}$ heteroduplex remained unaffected, the 1200-bp band disappeared (Fig. 4A, lane 2). When, after purification, DNA from this band was analyzed by gel electrophoresis, only a 600-bp band was found repeatedly (data not shown). In contrast, when DNA derived from the 600-bp band was incubated at high concentrations prior to electrophoresis, a 1200-bp band appeared. Again, heating to 70 °C and rapid chilling abolished that DNA form and led to a proportional increase in the amount of heteroduplex DNA (Fig. 4B). These findings strongly suggest that this DNA species represents a novel complex of DNA heteroduplexes.

**DISCUSSION**

The generation of DNA heteroduplexes in experimental procedures has been described previously. Methods for the detection of genomic mutations such as AMD (amplification and mismatch detection) are based on the annealing of sample-derived PCR products with nonmutated bona fide DNA (5). In these analyses, the heteroduplexes consist of DNA strands that differ only by single bases. The formation of heteroduplexes with long unpaired segments has been observed as a complication of quantitative PCR that use an artificial internal standard with partial sequence identity to the target DNA (6–8). Here we show that, in the analysis of genes with alternatively spliced transcripts, heteroduplex formation is a problem even where only qualitative aspects are concerned. We detected heteroduplexes after RT-PCRs of several alternatively spliced mRNAs (data not shown) such as those of the apoptosis regulator bcl-x (9) and of caspase 10 (10). Of particular importance is the appearance of the VEGF$_{145}$/VEGF$_{165}$ heteroduplex because, under standard agarose gel electrophoresis conditions, this complex has the same migration rate as the amplification product of a recently described minor splice variant, VEGF$_{145}$ (3). To investigate the significance of VEGF$_{145}$ expression, we avoided the formation of the VEGF$_{121}$ and VEGF$_{165}$ amplification products by selecting a 5′ primer that annealed within a part of exon 6 that is present in VEGF$_{149}$ and VEGF$_{145}$ but absent from VEGF$_{121}$ and VEGF$_{165}$. When cDNA from the A431 cells was used, the band corresponding to VEGF$_{145}$ was much less abundant than the VEGF$_{149}$ band. This observation contradicts results presented by Poltorak et al. (11) who employed an RT-PCR that allows for the formation of the VEGF$_{121}$/VEGF$_{165}$ heteroduplex. In light of our findings, it seems likely that the band obtained by Poltorak et al. (11) did not originate from VEGF$_{145}$ but rather represents a VEGF$_{121}$/VEGF$_{165}$ heteroduplex. Similar to the amplification products of VEGF$_{121}$ and VEGF$_{165}$, VEGF$_{145}$ must be expected to participate in heteroduplex formation. We could detect a heteroduplex of VEGF$_{145}$ and VEGF$_{165}$-derived DNA strands in the PCR reaction specific for those two splice forms but not when VEGF$_{121}$ and VEGF$_{165}$ amplification products were present (not shown). This was probably because of co-migration of small amounts of heteroduplexes containing VEGF$_{189}$ with other PCR products in agarose gel electrophoresis.

In addition to the DNA heteroduplex, a second DNA species was identified as a by-product of RT-PCR of alternatively spliced transcripts and was found to be a complex composed of two heteroduplexes. To our knowledge, this is the first report in which such a DNA heteroduplex-duplex is described. Based on the structural information obtained by S1 analysis of the heteroduplex, we conclude that this complex represents a novel DNA structure that is formed by base pairing of sequences exposed in single-stranded loops. Because of the limited length of the accessible complementary sequences, it is less resistant to denaturing conditions such as heating to 70 °C, but was nevertheless found to be stable at 4 °C as well as at room temperature. Because DNA heteroduplex structures are formed during recombination processes and bulging out of non-complementary segments has been proposed by current recombination models (12), it is tempting to speculate that interactions between DNA single-stranded loops could occur in vivo. Similar interactions between short single-stranded RNA loops have been implicated in the regulation of the replication of the bacterial plasmid ColE1 and in the initiation of dimerization of retroviral genomes (13, 14). Further studies are necessary to evaluate the importance of single-stranded DNA loops both in physiological and in experimental settings.

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**REFERENCES**
