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VNTR sequence on human chromosome 11p15 that affects transcriptional activity

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Abstract We found a variable number of tandem repeat (VNTR) sequence with 38-bp repetitive units in the promoter region of a gene of unknown function on human chromosome 11p15. Polymerase chain reaction amplification of this VNTR sequence using genomic DNA from 80 unrelated individuals revealed two common alleles, one with 10 (79% allelic frequency) and the other with 14 (14% allelic frequency) repetitive units, and two rare alleles with 22 (3%) or 30 (3%) repetitive units. We investigated whether differences in the length of this VNTR sequence would affect transcriptional activity of a heterologous promoter by transient transfection to NEC8, embryonal carcinoma cells derived from testis. The activity of the promoter was suppressed significantly when the VNTR region was cloned upstream, in a manner dependent on the number of repeats present in the VNTR sequence. The results implied that this polymorphic VNTR sequence might function as transcriptional regulator in 11p15, with differences in the number of repetitive units influencing efficiency of transcription of the gene lying downstream.

Key words VNTR · 11p15 · Transcriptional regulator · Polymorphism · Testis

Introduction

VNTR (variable number of tandem repeat) markers, also called single-copy minisatellites, were originally isolated from human DNA as highly informative restriction fragment length polymorphisms that were useful for linkage analyses because of their high degree of polymorphism among individuals (Nakamura et al. 1987). Evidence has lately emerged that some VNTR sequences may have significant roles in regulating transcription. For example, differences in monoamine oxidase A (MAOA) activity depend on the number of repeat units in a VNTR sequence located upstream of the *MAOA* gene (Sabol et al. 1998; Denney et al. 1999). Furthermore, Chevalier et al. (2001) have suggested that a four-allele VNTR sequence in the promoter region of the gene encoding cytochrome P450 prostacyclin synthase (*CYP8A1*) could account for individual differences in *CYP8A1* transcription in vivo, in response to interleukin-6.

Some investigators have reported that alleles at certain VNTR loci are associated with the prevalence of diseases such as ovarian cancer (Phelan et al. 1996; Weitzel et al. 2000), progressive myoclonus epilepsy of Unverricht-Lundborg type (Lafrenière et al. 1997; Alakurtti et al. 2000), and insulin-dependent diabetes mellitus (Lucassen et al. 1993; Pugliese et al. 1997; Vafiadis et al. 1997; Stead et al. 2000). A relationship may exist between the involvement of VNTRs in regulation of transcription and the association of some VNTR alleles with disease susceptibility.

In earlier work we found that cosmid clone cCI11p15-10 contained a VNTR sequence (Takita et al. 1992). Because genomic sequence analysis revealed that this VNTR sequence was located upstream of a gene corresponding to an expressed sequence tag, DKFZp434A0527, we chose to examine whether polymorphism of the repetitive sequence might influence the transcriptional activity. We show here that this VNTR sequence is polymorphic in the Japanese population, and that it can affect transcriptional activity of a promoter in vitro. Our data suggest that in vivo this VNTR sequence may influence transcription of DKFZp434A0527 in a manner dependent on the copy number of repetitive units present, causing differences among individuals as regards expression of this downstream gene, whose function is not yet known.

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Materials and methods

Genotyping

We synthesized two primers, V1 (5′-CCACCTGCCTACA TGGTATCTGC CTGAA-3′) and V3 (5′-GACAGCACT CAGAGCAGCCTAGAGAC-3′), on the basis of the DNA sequence from cosmid clone cCI11p15-10, to amplify genomic DNA containing the VNTR. We carried out polymerase chain reactions (PCRs) using genomic DNA from 80 unrelated Japanese individuals. Each PCR was performed in a volume of 25 μl containing 30 ng genomic DNA, 8.7 mM $MgCl_2$, PCR buffer (67 mM Tris at pH8.8, 16.6 mM $(NH_4)_2SO_4$, 6.7 μM ethylene-diamine tetraacetic acid, 10 mM β-mercaptoethanol), 10 pmol of each primer, 2.5 mM of each deoxyribonucleotide triphosphate, 10% dimethylsulfoxide, and 1.0 unit of Ex Taq DNA polymerase (TaKaRa Shuzo, Otsu, Japan), in a Gene Amp PCR9700 (Applied Biosystems, Foster City, CA, USA). The cycle conditions were 96°C for 4 min, followed by 35 cycles of 96°C for 30 s, 63°C for 30 s, and 72°C for 4 min. PCR products were separated by electrophoresis on 0.8% agarose gels, which were then stained with ethidium bromide.

Construction of reporter gene for luciferase assay

The four different VNTR alleles were cloned separately into the pGL3-Promoter vector (Promega, Madison, WI, USA), which had an SV40 promoter in front of a firefly luciferase gene. The sequence of each reporter construct was verified by the ABI 3700 autosequencer (Applied Biosystems).

Cell culture and transfection assay

A human testis-carcinoma cell line, NEC8 (obtained from RIKEN gene bank), was grown in RPMI1640 medium supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. The cells were plated on 60-mm dishes (2.0×10^5 cells/60-mm dish) and transfected 48 h later. pRL-TK vector (Promega), in which *Renilla* luciferase coding sequence was fused to the Herpes simplex virus thymidine kinase promoter region, was cotransfected as an internal control. We mixed each reporter vector with the internal control vector, and added the mixtures to 100 μl OPTI-MEM I medium (GIBCO BRL, Rockville, MD, USA) containing 6 μl FuGENE6 transfection reagent (Boehringer Mannheim, Mannheim, Germany). This medium was incubated for 15 min and then added to the NEC8 cultures. After 48 h of further incubation, the transfected cells were washed with phosphate-buffered saline and lysed with 500 μl of passive lysis buffer (Promega). Lysate proteins were retrieved in tubes and centrifuged briefly to remove cellular debris. Ten microliters of each extract was sequentially assayed for firefly and *Renilla* luciferase activities using the Dual-Luciferase Reporter System (Promega) according to the manufacturer's instructions. Luminescence measurements were carried out with a Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). Results were expressed as the ratio of activity of firefly to *Renilla* luciferase. All transfections were carried out in duplicate or triplicate, and each construct was tested in at least five independent experiments using separate preparations of plasmid DNA.

Statistical analysis

Transfection results were expressed as mean ± SD. Analyses of variance (ANOVA) and subsequent Scheffé's tests were used to determine significance of the differences in multiple comparisons. A difference in promoter activity among reporter constructs was considered significant statistically when the P value was less than 0.05.

Results

VNTR upstream of cDNA DKFZp434A0527

We previously reported that cosmid clone cCI11p15-10 contained a VNTR sequence (Takita et al. 1992). Sequence analysis of this cosmid clone disclosed a 38-bp consensus sequence of the repeating unit (CCTTGGTGAGGGGAC CTGGTGAGGGGACATGGGGGGGA) with some variations in length (38–41 bp) and DNA sequence from one unit to another (Fig. 1). A search for homology of this VNTR sequence with published genomic sequences revealed that it was located 125 nucleotides upstream of sequences corresponding to the 5′ end of a human cDNA, DKFZp434A0527 (Fig. 1).

To investigate polymorphism of this VNTR sequence, we synthesized a pair of PCR primers (V1 and V3, Fig. 1) corresponding to DNA sequences flanking the VNTR sequence, and performed PCR experiments using genomic DNA preparations from 80 unrelated individuals as templates. We detected four different alleles in this population sample: 750 bp, 900 bp, 1.2 kb, and 1.5 kb (designated as alleles A1, A2, A3, and A4, respectively). Estimated allelic frequencies are listed on Table 1; 11 of the 80 individuals were heterozygous at this locus. Sequence analysis of the 750 bp fragment (allele A1) indicated that this allele contained ten copies of the repetitive unit, but we were unable to determine the precise number of repetitive units in the other three alleles because DNA sequencing was unsuccess-

Table 1. Frequency and size of VNTR alleles at human chromosome 11p15

Allele	Allele size (kb)	Allele frequency (n = 160)	Number of alleles
A1	0.75	0.794	127
A2	0.9	0.144	23
A3	1.2	0.031	5
A4	1.5	0.031	5
Total	—	1.000	160

VNTR, variable number of tandem repeat

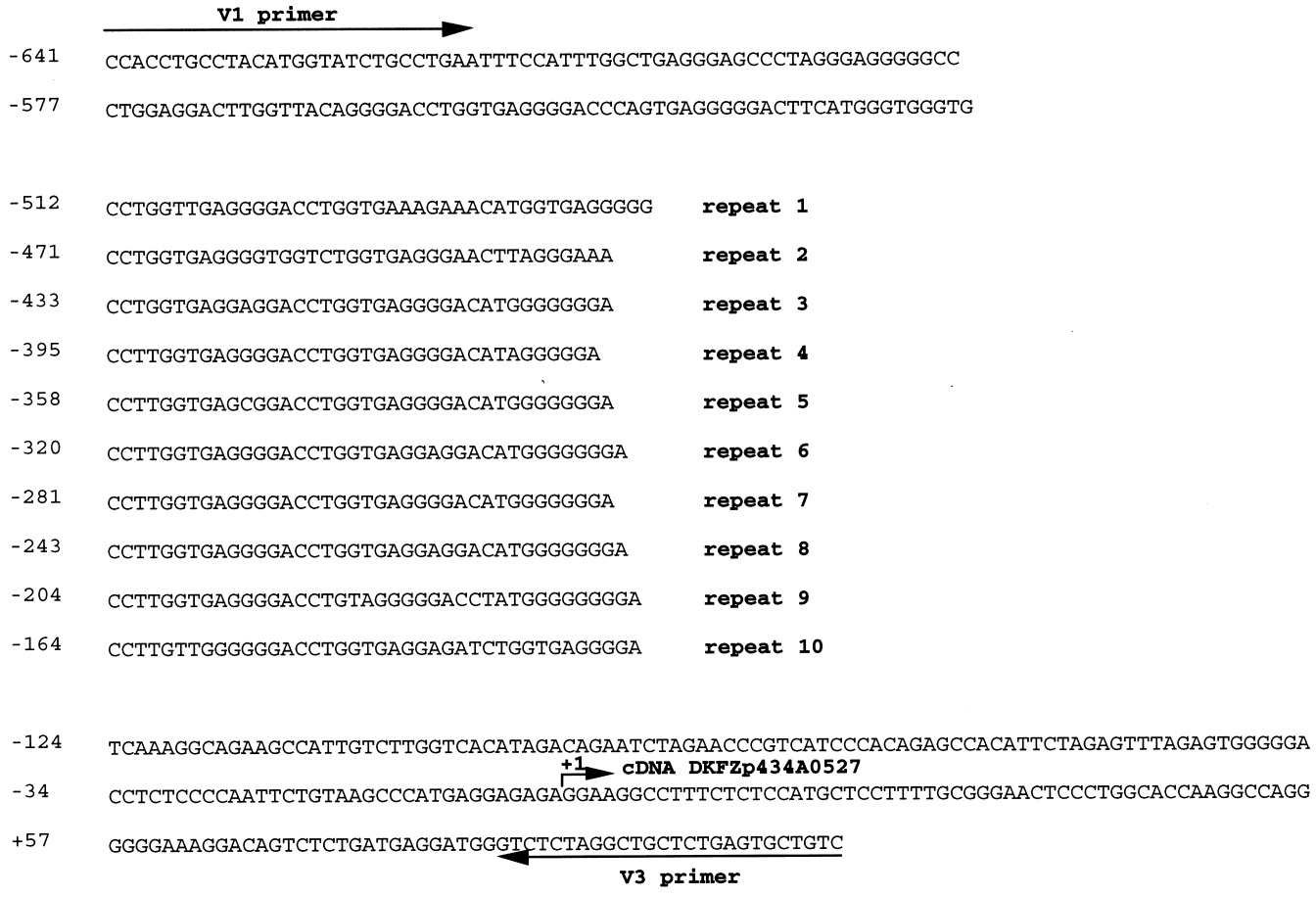

Fig. 1. Nucleotide sequence corresponding to allele A1 of the variable number of tandem repeat (VNTR) sequence, and the consensus sequence of the repetitive unit. *Arrows* indicate the locations of primers used to amplify the minisatellite. *Numbers* at the left indicate positions relative to the 5′ end of human cDNA DKFZp434A0527

ful, probably because of a very high proportion of G/C nucleotides.

In vitro analysis of the effect of the VNTR sequence on promoter activity

This VNTR sequence appeared to be located in the promoter region of DKFZp434A0527, although the function of this gene is unknown. Considering that this VNTR sequence was likely to be located in the promoter region (although the possibility of being located in an intron was not excluded) and that some VNTRs located near promoter regions or in introns are known to play significant roles in transcriptional regulation, we suspected that this VNTR sequence might have a similar influence on promoter activity. To verify this hypothesis, we cloned each of the four alleles of this VNTR sequence into a luciferase vector (pGL3-Promoter vector) and examined the effect that different numbers of repeat units would have on transcriptional activity in the reporter assay after transient transfection into a testis-carcinoma cell line, NEC8.

All constructs that contained one of the VNTR alleles suppressed the transcriptional activity of the SV40 promoter present in the pGL3-Promoter vector. We also made a construct containing a 750bp genomic DNA fragment without any repetitive sequences and performed the same experiment as a control, but observed no effect on the transcriptional activity of the reporter gene (Fig. 2). There were distinct differences in transcriptional activity between this control construct and each VNTR allele ($P < 0.0001$). The greater the number of repetitive units present, the more the transcriptional activity was suppressed; i.e., the rare, longer alleles (A3 or A4) seemed to suppress the promoter activity of the reporter gene more than did the major, shorter alleles (A1 and A2) (A4 vs. A1 and A2; $P < 0.0001$; A3 vs. A1; $P = 0.0002$; A3 vs. A2; $P = 0.0065$). On the other hand, the difference in promoter activities between alleles A1 and A2 was not statistically significant ($P = 0.8340$), nor was the difference between alleles A3 and A4 ($P = 0.3866$).

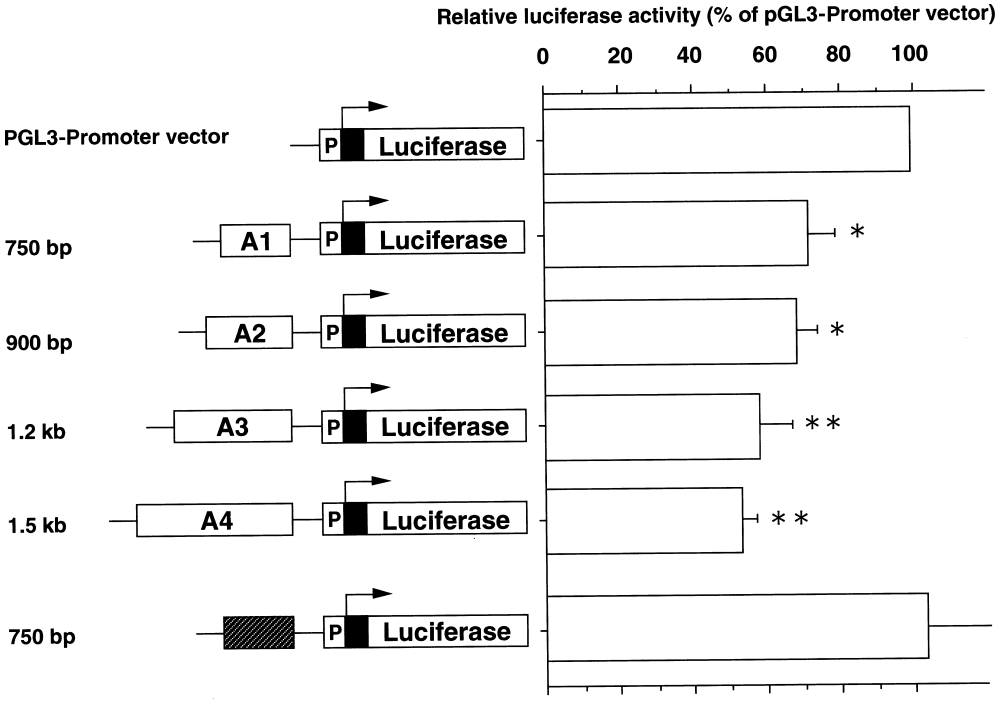

Fig. 2. Influence of the VNTR sequence on promoter activity in transiently transfected NEC8 cells. The diagram indicates the luciferase activity of each VNTR allele, corrected for transfection efficiency and recorded relative to that of the pGL3-Promoter vector alone. Mean ± SD values for at least five independent experiments are shown. *P* indicates SV40 promoter that is present in the pGL3-Promoter vector. The *hatched box* denotes a 750-bp genomic DNA fragment containing no repetitive sequences. $*P < 0.0001$, alleles A1 and A2 vs. pGL3-Promoter vector or control; $**P < 0.0001$, alleles A3 and A4 vs. pGL3-Promoter vector or control, and $P < 0.05$ for alleles A3 and A4 vs. alleles A1 and A2

Discussion

We reported previously that six cosmid clones on human chromosome 11p15 contained VNTR sequences (Takita et al. 1992). DNA sequencing of these VNTRs and comparisons with genomic sequences disclosed that one VNTR sequence was likely to be located in the promoter region of a gene of unknown function, designated DKFZp434A0527. Reverse transcriptase-PCR experiments using this human cDNA as a template revealed that the gene was expressed in human testis and kidney (data not shown).

In a Japanese population sample of 160 chromosomes, we detected four different alleles of this VNTR sequence. The most common allele (A1) contained ten copies of the repetitive unit. Although we were unable to obtain the complete DNA sequences of the other three alleles for technical reasons, the copy numbers of the repetitive units were speculated to be 14 (A2), 22 (A3), and 30 (A4) on the basis of fragment lengths estimated by gel electrophoresis.

In vitro analysis of the effect of the different alleles on transcriptional activity was carried out using six plasmid clones, four of which contained a single allele of the VNTR sequence. Each clone was transfected into NEC8 cells and luciferase activity was measured. Alleles A3 and A4, corresponding to larger numbers of repeat units, suppressed transcriptional activity more significantly than did the shorter alleles (A1 and A2). When we compared the 750 bp DNA fragment containing this VNTR sequence with 750 bp of genomic DNA containing no repeat sequence, only the fragment containing the VNTR sequence was able to suppress transcription. Because we obtained similar results in experiments using HeLa cells (data not shown), we suspected that this VNTR sequence was likely to function as a transcriptional regulator in a nontissue specific manner.

The mechanism by which the major alleles (A1 and A2) and minor alleles (A3 and A4) influence transcriptional activity to different extents is unknown. Some transcription factors are known to bind to VNTR sequences. For example, members of the rel/NF-kB family of transcription factors bind VNTR sequences present in the 3′ flanking region of the *HRAS* gene; the consensus sequence of the repeat units in that VNTR sequence is highly homologous to the binding site of rel/NF-kB proteins (Trepicchio and Krontiris 1992). The same authors (1993) reported that although the VNTR sequence present in the human immunoglobulin heavy chain gene (*IGH*), which contains a sequence similar to the motif for binding the myc/HLH transcription factor, had no enhancer activity, it was able to significantly suppress transcriptional activity of the adenovirus major late promoter. We speculate that some transcription factor(s) might bind to the repetitive sequence reported here, although at present we have no data to support this idea.

Associations of certain VNTRs with the risk of diseases have also been documented (reviewed by Nakamura et al. 1998). The presence of one or two of the rare alleles of the *HRAS* VNTR sequence in a single individual appear to be associated with a higher risk of cancers in multiple tissues, e.g., colon, breast, and urinary bladder (Krontiris et al. 1993). Phelan et al. (1996) suggested that the status of the *HRAS* VNTR sequence might influence the penetrance of ovarian cancer in individuals who carried a mutation in the breast-cancer susceptibility gene *BRCA1*. Considering that the novel VNTR sequence reported here seems to be located in the promoter region of a cDNA (DKFZp434A0527) that is expressed in testis and kidney,

and is able to regulate in vitro transcriptional activity in a manner that depends on the number of repeats present, one or two of the rare VNTR alleles in a single individual might reduce expression of this gene and influence the individual's susceptibility to diseases involving testis or kidney.

In conclusion, our study has demonstrated polymorphism of this VNTR sequence among Japanese individuals and shown that its alleles could be ranked in two classes according to allele frequency and the effect on transcriptional activity. We have not determined the mechanism responsible for differences in transcriptional effect pertaining to each allele, but it is possible that this VNTR sequence might contain the binding site of a transcriptional regulator and the numbers of such units could thereby influence the efficiency of transcription.

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