3′-Untranslated region of the type 2 bradykinin receptor is a potent regulator of gene expression

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Zamorano, Rocio, Sunil Suchindran, and James V. Gainer. 3′-Untranslated region of the type 2 bradykinin receptor is a potent regulator of gene expression. Am J Physiol Renal Physiol 289:F456–F464, 2005.—Regulation of the constitutively expressed type 2 bradykinin (B2) receptor, which mediates the principal actions of bradykinin, occurs at multiple levels. The goal of the current study was to determine whether the human B2 3′-untranslated region (UTR) has effects on gene expression, with particular focus on the variable number of tandem repeats (B2-VNTR) polymorphic portion of the 3′-UTR and its flanking AU-rich elements (AREs). When inserted downstream of the luciferase coding region of the pGL3-Promoter vector, the B2-VNTR reduced reporter gene activity by 85% compared with pGL3-Promoter alone (promoter control; P < 0.001), an effect that was not appreciably affected by mutation of the flanking AREs. The negative regulatory effects of the B2-VNTR region were position and orientation dependent and strongly positively correlated with the number of tandem repeats in the B2-VNTR region (r = 0.85, P < 0.001). With respect to mechanism, quantitative RT-PCR revealed that the B2-VNTR mRNA level was 32% of that of promoter control (P = 0.008), whereas the number of polyadenylated transcripts was 4% (P = 0.02). In contrast, the mRNA half-life of the B2-VNTR was increased (B2-VNTR: 14.9 vs. promoter control: 12.2 h, P = 0.009). Transient transfection of human kidney-derived tsA201 cells with the B2-VNTR construct increased transcribed and translated mRNA by 43% (P < 0.05), supporting an endogenous B2 receptor-regulatory capacity of the B2-VNTR. In conclusion, these results identify novel posttranscriptional effects of the B2-VNTR region to act as a potent negative regulator of heterologous gene expression and support the notion that the bradykinin B2 3′-UTR may impact endogenous receptor regulation.

Seventy years ago, John Watson made a fateful distinction between the genotype and the phenotype of an organism. Today, in the context of gene expression, the distinction between genotype and phenotype is more complex. For example, gene regulation is a complex process that involves multiple levels of regulation. The regulation of B2 receptor expression can be influenced by both transcriptional and posttranscriptional mechanisms. The 3′-UTR is a region of the B2 receptor mRNA that can affect gene expression. Several studies have shown that the 3′-UTR can act as a negative regulator of gene expression by reducing the stability of the mRNA or by affecting the translation of the mRNA. The goal of this study was to determine the effects of the human B2 3′-UTR on gene expression.

BRADYKININ HAS AN INTEGRAL relevance to a range of biological processes including vasodilatory control (25, 26), sodium homeostasis (1), fibrinolysis (6), pain (7, 12), allergy (19, 31), and inflammation (7, 19). The principal actions of bradykinin and the kinin system are mediated by the constitutively expressed type 2 (B2) receptor, which has extensive tissue distribution, notably in the kidney, heart, skin, and peripheral vasculature (17, 26). The use of bradykinin receptor antagonists specific for the B2 receptor as well as knockouts and transgenic mice has established beneficial effects of kinins to reduce blood pressure (15, 25), oppose vasoconstrictive and growth effects of ANG II (20, 35), and stimulate fibrinolysis (6). Other studies support the involvement of the kinin system in the pathogenesis of asthma (19, 31), arthritis (10), and angioedema (19).
focus on the VNTR polymorphic portion (B2-VNTR) of the 3'-UTR including the flanking ARE segments. In this report, we provide evidence that the B2 3'-UTR harbors a functional element within the B2-VNTR region that potently regulates gene expression which occurs through a non-ARE-dependent mechanism. We demonstrated that the B2-VNTR, which is not conserved among species, acts in a position- and orientation-dependent fashion in a reporter plasmid to reduce gene expression in human and nonhuman cell lines. We further showed that the transfection of the minimal B2-VNTR element in a human kidney-derived cell line, which expresses the bradykinin receptor, modulates the regulation of the endogenous B2 receptor.

MATERIALS AND METHODS

3'-UTR constructs. Segments of the human B2 receptor 3'-UTR were PCR-amplified using oligonucleotide primers containing flanking SacII and NdeI recognition sequences. For antisense constructs, the flanking restriction endonuclease sequences for the primers were interchanged. The PCR products were gel-purified and ligated downstream of the firefly luciferase coding region of the pGL3-Promoter vector (Promega, Madison, WI). The XbaI restriction site in this vector was reengineered with a linker segment to allow unidirectional insertion of the 3'-UTR. The pGL3-Promoter vector backbone was chosen because it contains the SV40 promoter without enhancers; therefore, changes in luciferase activity can be attributed to the effect of 3'-UTR inserts. The following primers were used to amplify the B2-VNTR region: forward: 5'-ctcagcaacagaggtttg-3'; reverse: 5'-GGTCAGGATTATGGGCTCTT-3'. The sequence of the most commonly occurring variant (43 repeats) of the B2-VNTR region was used in this experiment (5). All constructs were confirmed by direct sequencing.

Cell culture/transient transfections. Human kidney-derived tsA201 cells, a subclone of the HEK293 cell line, were cultured in a defined medium made by mixing equal parts of DMEM and CooN's modified medium with 5% CO2. tsA201 cells were plated at 50–60% confluence in 24-well dishes 24 h before transfection. Pilot studies were performed using site-directed mutagenesis performed according to the manufacturer’s instructions (Stratagene, La Jolla, CA). Mutagenesis oligonucleotide primers (1st ARE forward: 5'-gggcagcactcaaccttgataaagtgaAGGGTatgctgg-3' and 2nd ARE forward: 5'-gggcttcgaagccgtaagagaAACGGTTacagctggagacc-3'; mutated bases underlined) were added to purified plasmid DNA and extended during temperature cycling using Pfu Turbo DNA polymerase. The resultant product was digested with DpnI and transformed into DH5.1-α cells. Mutagenesis products were confirmed by direct sequencing.

Measurement of luciferase activity. To determine whether the B2 3'-UTR could confer transcriptional or posttranscriptional control of gene expression in a heterologous reporter system, we compared luciferase activity among chimeric luciferase constructs which contained either 1) the B2-VNTR which encompasses the midsegment of the B2 3'-UTR and flanking class I AREs (defined as the occurrence of an AUUUA pentamer in a non-U-rich region), 2) the 3'-UTR from the smooth muscle myosin light chain (SMLMC) (used as a length control), or the 3) insertion-less pGL3 Promoter (designated promoter control).

Luciferase reporter assays were performed using the Dual-Glo Luciferase Reporter Assay System (Promega). At the time of harvest, the culture medium was removed, and cells were washed with phosphate-buffered saline. Passive lysis buffer (100 µl of 1× buffer) was added to each well, and plates were placed in a shaking incubator for 15 min at room temperature. For additional lysis, two freeze-thaw cycles were performed in which the cells were frozen to −80°C. The lysate was placed in a 96-well luminometer plate (Packard Bioscience, Billerica, MA) with an equal volume of Dual-Glo Luciferase Reagent and incubated for 10 min. Firefly luciferase luminescence was measured. Before measurement of Renilla luciferase (pRL-TK vector, Promega), 100 µl of the Stop and Glo reagent (Promega) were added to each well to quench the firefly luciferase reaction. Renilla luciferase luminescence was measured after an incubation of 10 min. All luciferase measurements represent an average of readings performed in triplicate. Fresh reagents were used for entire sets of samples and their duplicates to ensure equivalent reaction conditions. Relative firefly luciferase light output was normalized by Renilla luciferase output after appropriate subtraction of background light output. In most cases, data points represent the means ± SE of two or three iterations of three to seven independent experiments.

Mutation assay. In the experiments regarding the role of the AREs in gene expression, the core AUUUA pentamer of each element was altered using site-directed mutagenesis performed according to the manufacturer’s instructions (Stratagene, La Jolla, CA). Mutagenesis oligonucleotide primers (1st ARE forward: 5'-gggcagcactcaaccttgataaagtgaAGGGTatgctgg-3' and 2nd ARE forward: 5'-gggcttcgaagccgtaagagaAACGGTTacagctggagacc-3'; mutated bases underlined) were added to purified plasmid DNA and extended during temperature cycling using Pfu Turbo DNA polymerase. The resultant product was digested with DpnI and transformed into DH5.1-α cells. Mutagenesis products were confirmed by direct sequencing.

Levels of chimeric luciferase transcripts were determined using real-time quantitative reverse transcriptase PCR using SYBR Green chemistry. Total RNA was extracted from tsA201 cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to instructions from the manufacturer. To eliminate amplification of reporter plasmid DNA and genomic DNA, total RNA was treated with DNase RQI (Promega, 10 U) at 37°C for 30 min, followed by resolulation of RNA with phenol/chloroform treatment. RNA (1.0 µg) was reverse transcribed with SuperScript II RNase H reverse transcriptase (Invitrogen) using either random hexamer or oligo dT primers. After first-strand synthesis, the cDNA was quantified using the DNA-binding dye SYBR green I (Qiagen, Valencia, CA). Fluorescence was detected using an iCycler iQ sequence detection system (Bio-Rad, Richmond, CA). Luciferase amplification primers were forward: 5'-GGCTGGAAGGTCGGCTGCTG-3' and reverse: 5'-ACACCTGGCGTGAAGATGTG-3'. Renilla luciferase primers were forward: 5'-ATGGTGAATGCGCTGAT-3' and reverse: 5'-CAATGCGTTCCACGAAGA-3'. Amplification primers for human glyceraldehyde-3-phosphate (GAPDH) were 5'-TCTGTCTGTTCGTG-3' for the forward primer and 5'-GAAATGGTGATGGGAGGGC-3' for the reverse primer. For detection of the B2 receptor, the following primers were used: forward: 5'-TCAATGT-TTCTGCTGGTCTG-3'; reverse: 5'-AAAAAGTCCGTTAGAG-GTGG-3'.
Fig. 1. Genomic structure of the B2-VNTR. Shown is the purine-rich structure of the tandem repeats polymorphism and the flanking AU-rich elements (AREs).

Results

Characteristics of the B2-VNTR region. As shown in Fig. 1, the B2-VNTR contains the purine-rich tandem repeat polymorphism which is composed of four alleles, the most common of which is the 43-repeat allele (5), and also includes the flanking class I flanking AREs which contain the core AUUUUA pentad (upstream: AAUAAUUAUUA; downstream: AAAAUUUAUCAU). With respect to species conservation, the B2-VNTR segment is also found in the chimpanzee (P. troglodytes) and contains 32 repeats but otherwise shows little conservation among species (Table 1), such that no identifiable VNTR exists in the B2 3′-UTR for the rat (R. norvegicus), mouse (M. musculus), or dog (C. familiaris). In humans and chimpanzees, the B2-VNTR segment is a purine-rich pocket (68 and 64%, respectively) in the 3′-UTR which otherwise has a purine percentage of 54%. Despite the lack of sequence identity in the B2 3′-UTR among species with respect to the VNTR, a purine-rich tandem repeat structure with considerable similarity to the B2-VNTR was identified in the rat, mouse, and dog in alternate chromosomal locations.

B2 3′-UTR is a potent regulator of gene expression. The tsA201 cells were transiently transfected with luciferase vectors (pGL3-Promoter) in which the B2-VNTR or SMMLC 3′-UTRs were inserted downstream of the luciferase coding region. Renilla-normalized luciferase activity for each construct was compared with the insertion-less pGL3-Promoter vector (promoter control). As shown in Fig. 2, the construct containing the B2-VNTR reduced luciferase expression by 85% compared with promoter control (−85 ± 1%, Z = −4.4, P < 0.001). Gene expression of the construct containing the SMMLC 3′-UTR, used as a length control, was increased over that of promoter control (31 ± 1%; Z = −4.0, P < 0.001).

AREs in the B2 3′-UTR do not affect gene expression. To determine the importance of the AREs to gene expression, one or both AREs were mutated by site-directed mutagenesis. As shown in Fig. 3, compared with the nonmutated B2-VNTR construct, normalized luciferase activity was minimally different for constructs in which the first (difference = −2%, Z = −2.9, P = 0.004) or both AREs were mutated (difference = −3%, Z = −2.9, P = 0.004). These data suggest that the AREs do not have appreciable functional effects on gene expression in the context of the B2 3′-UTR.

Effects of B2-VNTR on gene expression are position and orientation dependent. To assess whether the B2-VNTR region could operate in the inverse orientation, the reverse complement of the B2-VNTR segment was cloned into the pGL3-Promoter vector downstream of the luciferase coding region (B2-VNTR-INVERSE). This construct reduced luciferase activity to 56% of promoter control (P < 0.001) or approximately two-thirds of the reduction attributable to the sense orientation of the B2-VNTR construct (Fig. 4).

To determine whether the location of the B2-VNTR in the luciferase reporter plasmid influenced its capacity to alter reporter gene expression, both the B2-VNTR and B2-VNTR-INVERSE segments were inserted into the multiple cloning region (MCR) adjacent to the SV40 promoter of the pGL3-Promoter vector. As shown in Fig. 4, transcription attributable to these constructs was not significantly different from that of

Table 2. Tandem repeat segments similar to the B2-VNTR region in other species

<table>
<thead>
<tr>
<th>Species</th>
<th>Chromosome #</th>
<th>Repeat Sequence</th>
<th>Repeat, #</th>
<th>Purines, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat (R. norvegicus)</td>
<td>14</td>
<td>TGAGGAGCTAGAACC</td>
<td>33–43</td>
<td>0.66</td>
</tr>
<tr>
<td>Mouse (M. musculus)</td>
<td>15</td>
<td>TGAGGAGCTAGAACC</td>
<td>32</td>
<td>0.68</td>
</tr>
<tr>
<td>Dog (C. familiaris)</td>
<td>8</td>
<td>TGAGGAGCTAGAACC</td>
<td>32</td>
<td>0.68</td>
</tr>
</tbody>
</table>

Shown are the species, chromosomal location, and tandem repeat sequence and number of repeats for a tandem repeat structure similar to the B2-VNTR but not located within the B2 3′-UTR.
promoter control, nor was there a difference when the B2-VNTR was inserted in a position downstream of the SV40 late polyA signal in the pGL3-Promoter vector (Fig. 4). These results support that contention that the effects of the B2-VNTR on transcription are strictly position dependent but are also orientation dependent such that the maximal effect is observed in the sense orientation.

Multiple cis-elements are associated with a reduction in gene expression by the B2 3′-UTR. To test the hypothesis that cis-acting elements, other than the AREs, within the B2-VNTR region mediated the effects on gene expression, we compared luciferase expression among a series of 3′-UTR deletion constructs (Fig. 5). The maximal reduction of gene expression was observed with the B2-VNTR, which includes upstream and downstream flanking regions. The upstream flanking region tested alone significantly reduced gene expression (−58%; $Z = -3.1; P = 0.002$), whereas the effect of the downstream flanking region was modest (−27%; $Z = -3.1; P = 0.002$) compared with promoter control. The number of tandem repeats in the B2-VNTR region correlated positively with the reduction in gene expression ($r = 0.85, P < 0.001$). Taken together, these data support the presence of elements both within and upstream of the B2-VNTR region that reduce gene expression but suggest that the regulatory potential of this region depends on a critical number of tandem repeats.

Fig. 2. Effect of B2 3′-untranslated region (UTR) on luciferase reporter gene expression. B2 3′-UTR constructs containing either the B2-VNTR (variable number of tandem repeats) region or the smooth muscle myosin light chain 3′-UTR (SMMLC; used as a length control, 850 nt) were compared with pGL3-P (promoter control vector without insert). Luminometer measurements (±SE) for this and subsequent experiments are expressed as fold of promoter control in relative light units of firefly luciferase normalized by Renilla luciferase (index of transfection efficiency) and represent an average of 2–7 independent experiments performed in duplicate for each construct with luciferase activity measured in triplicate.

Fig. 3. Effect of ARE on gene expression. Reporter gene expression was compared between B2-VNTR constructs in which none, one, or both ARE sequences were mutated.
B2-VNTR is associated with reduced mRNA levels but not accelerated mRNA degradation. To determine whether accelerated mRNA decay can contribute to the observed reduction in luciferase activity associated with the B2-VNTR, real-time quantitative PCR was used to compare mRNA levels (normalized log SQ mean) between the B2-VNTR and promoter control at various timepoints after the addition of transcriptional inhibitor actinomycin D, which was initially added at 48 h following transfection, denoted as time 0 (Fig. 6). These data were normalized by Renilla to control for transfection efficiency. With respect to mRNA decay, the calculated half-life of the B2-VNTR construct was significantly greater than that of the promoter control (B2-VNTR: 14.9 vs. promoter control: 12.2 h, Z = −2.6; P = 0.009). These data were qualitatively similar to that obtained for the respective constructs after transfection in Madine-Darby canine kidney (MDCK-2) cells (data not shown). These data do not suggest that an accelerated degradation of mRNA contributed to the observed reduction in reporter gene expression associated with the B2-VNTR.

B2-VNTR is associated with decreased polyadenylation of heterologous transcripts. To determine more precisely whether a posttranscriptional mechanism contributes to the effect of B2-VNTR to reduce luciferase activity, we measured total RNA and polyadenylated mRNA levels for each of the constructs. As summarized in Table 3, total RNA (normalized SQ

Fig. 4. Effect of orientation and position of the B2-VNTR on gene expression. Reporter gene expression was assayed in constructs in which the B2-VNTR region was inserted in either the sense or antisense (B2-VNTR-INVVERSE) orientations in either the 3′-UTR, adjacent to the SV40 promoter multiple cloning region (MCR), or downstream of SV40 late polyA signal of the pGL3-Promoter plasmid vector and compared with promoter control.

Fig. 5. Deletion analysis of the B2-VNTR and effect of number of tandem repeats. Luciferase activity was compared among various deletion constructs of the B2-VNTR region. Thick dashed line represents the occurrence of 3 successive segments nearly identical to the 5-repeat fragment. Luminometer measurements (±SE) are expressed as the fold of promoter control in relative light units of firefly luciferase normalized by Renilla luciferase (index of transfection efficiency) and represent an average of 2–7 independent experiments performed in duplicate for each construct with luciferase activity measured in triplicate.
mean) for the B2-VNTR construct was 32% of that of promoter control (B2-VNTR vs. promoter control: 0.27 ± 0.12 vs. 0.86 ± 0.04; P = 0.008) at 48 h following transfection. The B2-VNTR construct was associated with reduced polyadenylation efficiency which resulted in a polyadenylated B2-VNTR mRNA level that was 4% of promoter control (B2-VNTR vs. promoter control: 0.03 ± 0.01 vs. 0.74 ± 0.11; P = 0.02). These results support the notion that the B2-VNTR affects processes involved in effective 3′-end formation of mRNA.

**EMSAs of B2-VNTR using nuclear and cytoplasmic protein extracts.** To assess the capacity of the B2-VNTR region to bind proteins, we performed gel shift assays in which a biotinylated minimal B2-VNTR element (the 203-bp segment designated 12-REPEATS in Fig. 5) was exposed to both nuclear and cytoplasmic tsA201 protein extracts. As shown in Fig. 7A, the addition of the minimal B2-VNTR element to the nuclear proteins resulted in a specific band shift that was not observed with the addition of a comparably sized nonspecific competitor. In contrast, the pattern of banding was similar between the B2-VNTR and the competitor using cytoplasmic protein extracts (Fig. 7B). These data suggest that the regulatory effect associated with the B2-VNTR may involve protein binding which occurs in the nuclear compartment.

**Native B2 receptor transcription is increased with cotransfection of the B2-VNTR.** To determine whether the B2 3′-UTR has the potential to affect endogenous B2 receptor transcription, the B2-VNTR and promoter control were transiently transfected separately (n = 5) in tsA201 cells, and the native B2 receptor mRNA was measured using real-time quantitative PCR. Following normalization with GAPDH, native B2 mRNA was increased by 43% (Z = −2.0; P < 0.05) in cells transfected with the B2-VNTR compared with those transfected with promoter control (Table 4). These data suggest that the B2 3′-UTR has the capacity to influence the regulation of the endogenous B2 receptor.

### DISCUSSION

In this study, we demonstrated that the human B2 3′-UTR harbors elements that negatively regulate gene expression. We focused on the portion of the 3′-UTR (B2-VNTR) that contained a tandem repeats-type polymorphism that is flanked by AREs. Many studies demonstrated the importance of AREs as putative mediators of posttranscriptional and translational control (37, 38). By binding trans-acting factors, such as hnRNPs and Hu proteins, AREs exert regulatory control of gene expression through rapid degradation of mRNAs and/or repression of translation (3) and operate in a context- and stimulus-specific manner (38). In the current study, however, we detected no effects of the AREs contained in the B2-VNTR region to mediate or modify the observation of reduced luciferase expression. In contrast, we found that the VNTR segment of the B2 3′-UTR itself accounted for the significant negative regulatory effect on reporter gene activity.

Regulatory sequences downstream of the promoter have the capacity to modulate transcriptional (8, 34), posttranscriptional (24), and even translational activity (21, 32, 39, 40). For example, Carrion et al. (8) demonstrated the existence of a downstream regulatory element (DRE) sequence that mediates position-dependent, but orientation-independent, repression of prodynorphin gene transcription from its location in the 3′-UTR. Sanz et al. (34) reported an example of a DRE sequence, which is located downstream of the open reading frame in the 3′-UTR of the apoptotic hrk gene, that on binding a repressor protein results in transcriptional silencing. Analysis of the DRE sequence revealed a core GTCA sequence, similar to that found in CRE and AP-1 elements, which could also function in an inverse orientation (22). In contrast to this situation, we found no evidence for a regulatory effect of a segment containing fewer than five copies of the core tandem repeat sequence of the B2-VNTR; however, the degree of repression was strongly positively correlated with the number of tandem repeats (Fig. 5). Using a deletional analysis, we determined that the capacity of the B2-VNTR to repress gene expression was largely localized to the proximal portion of the element, and its effects, like the DRE sequence, were position dependent, but in contrast to the DRE sequence were more marked in the sense orientation.

To address more specifically the level at which the B2-VNTR acts to regulate gene expression, quantitative RT-PCR revealed that the total RNA level of the B2-VNTR construct was 32% of that of promoter control. With respect to the genomic structure of the B2 receptor, the B2-VNTR occurs in the untranslated portion of the terminal exon, suggesting the

Table 3. Effects of B2-VNTR on RNA transcript levels

<table>
<thead>
<tr>
<th>Construct</th>
<th>Total RNA (Normalized SQ Mean)</th>
<th>Ratio of Total RNA (B2-VNTR/Control)</th>
<th>Polyadenylated RNA</th>
<th>Ratio of Polyadenylated RNA (B2-VNTR/Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2-VNTR</td>
<td>0.27±0.12</td>
<td>0.32</td>
<td>0.03±0.01</td>
<td>0.04</td>
</tr>
<tr>
<td>Promoter control</td>
<td>0.86±0.04</td>
<td></td>
<td>0.74±0.11</td>
<td></td>
</tr>
</tbody>
</table>

Shown are total RNA and polyadenylated RNA levels for both the B2-VNTR construct and pGL3-Promoter (promoter control) and the ratio of total and polyadenylated RNA for the respective constructs. Results were normalized by cotransfected Renilla luciferase.
Fig. 7. Nuclear and cytoplasmic electrophoretic mobility shift analysis (EMSA) of the B2-VNTR. EMSA was performed using a biotin-labeled minimal B2-VNTR probe (203 nt; 12-repeat construct shown in Fig. 6). A and B: labeled probe alone (free probe) is shown in lane 1. Lane 2 corresponds to the labeled probe incubated with tsA201 nuclear (A) and cytoplasmic (B) extracts. For competition experiments, nonlabeled B2-VNTR (lane 3) or nonspecific competitor (lane 4) was added in 10–25 × molar excess to protein extracts and incubated followed by the addition of labeled probe. Protein-DNA complexes were resolved by 5% polyacrylamide gel electrophoresis, transferred to a nylon membrane, and visualized on X-ray film.

Table 4. Effects of B2-VNTR on endogenous B2 receptor RNA transcript levels

<table>
<thead>
<tr>
<th>Construct</th>
<th>B2 Receptor RNA (B2-VNTR/Control Transfection)</th>
<th>Ratio of B2 Receptor RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2-VNTR</td>
<td>1.28±0.11</td>
<td>1.40</td>
</tr>
<tr>
<td>Promoter control</td>
<td>0.92±0.15</td>
<td></td>
</tr>
</tbody>
</table>

Shown is the RNA level for the B2 receptor for the B2-VNTR construct and pGL3-Promoter (promoter control) and the ratio of B2 receptor RNA after transfection with each of these constructs. Results were normalized by simultaneous measurement of GAPDH RNA levels.

Several aspects of B2 receptor regulation have been well characterized. In studies of the rat B2 promoter, factors such as cAMP (9, 11, 29) can transcriptionally increase B2 receptor expression in a cell-specific manner and may contribute to the cytokine stimulation of the B2 receptor by interleukin-1β (36). Transcriptional control of the B2 receptor may have particular relevance in the developing kidney where a complex regulatory role of p53 has been demonstrated (33). A transcriptional silencing mechanism in the rat B2 promoter has also been reported that is mediated by a putative SRE-1 site (2). In the context of inflammation, Lung et al. (24) characterized an upregulation of human B2 receptor mRNA in response to interferon-γ that was dependent on both transcriptional and posttranscriptional components.

In the current study, we addressed the potential for the B2-VNTR to affect endogenous regulation of the B2 receptor. Cotransfection of the B2-VNTR in cells expressing the B2 receptor (tsA201) was associated with a 43% increase in native B2 receptor mRNA level. These results coupled with the gel-shift data that show binding of nuclear proteins to the B2-VNTR support the speculation that a nuclear protein(s) bound by the B2-VNTR has the capacity to negatively regulate events leading to the processing of mature mRNA transcripts.

It is unknown whether the mechanism by which the B2-VNTR segment regulates gene expression is applicable to other species or other genes. With respect to comparative genomics, the B2-VNTR segment in the human B2 receptor is similar to that of the chimpanzee but displays little sequence conservation with the rat, mouse, or canine B2 receptor sequences. Remarkably, as shown in Table 2, a tandem repeat structure which bears considerable similarity to that of the B2-VNTR occurs in these latter species in alternate chromosomal locations, the significance of which awaits further study.

In summary, we identified a novel effect of the B2 3′-UTR to act as a potent negative regulator of heterologous gene expression and provided data to support that the B2-VNTR modulates transcript-processing events such as polyadenylation which impact the efficiency of 3′-end formation. In addition, we identified the capacity for the B2-VNTR element to

possibility that the B2-VNTR could influence the 3′-end formation of mRNA. In support of this contention, our data showed that the B2-VNTR region had a marked effect on the efficiency of polyadenylated RNA transcripts was 4% compared with control levels. Given the complexity of transcriptional regulation, additional studies are necessary to rule out the contribution of other transcript processing events. Taken together, these results are consistent with the hypothesis that the B2-VNTR acts at a point before the successful transport of transcripts into the cytoplasm. Indeed, our gel shift data showed that the B2-VNTR binds nuclear but not cytoplasmic protein extracts and supports the notion that the mechanism of the B2-VNTR to repress reporter gene expression occurs at a pretranslational level.

Somewhat surprisingly we found that the B2-VNTR was associated with increased mRNA stability (Fig. 6), which suggests the possibility that the B2-VNTR could also affect translational events. Several studies demonstrated that untranslated regions, notably the 3′-UTR, can critically impact translational regulation (21, 32, 39, 40). By virtue of the potential to bind trans-acting factors, in some cases directed by specific structural features, the 3′-UTR can act to either enhance or repress translation, the latter of which is exemplified in the 3′-UTR of the 15-lipoxygenase (15-lox) gene. Translational repression occurs in 15-lox by a mechanism that involves protein binding of heteronuclear RNP K and α-CP to a motif that is comprised of a pyrimidine-rich 19 nt tandem repeat located in the 3′-UTR (28).
participate in the regulation of the native B2 receptor in a human kidney-derived cell line. Studies involving further fine localization of regulatory sequences in the B2-VNTR and identification of binding proteins will help address the relevant questions of whether the B2 3′-UTR contributes to the endogenous regulation of the B2 receptor in vivo and participates in kinin-dependent pathophysiology and also determine the generalizability of this 3′-UTR-based mechanism to the regulation of other genes.

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