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ORIGINAL PAPER

Polymorphisms in the *Paan-AG* promoter influence NF-κB binding and transcriptional activity

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Abstract The human leukocyte antigen-*G* (*HLA-G*) gene encodes a protein that is highly expressed at the human maternal–fetal interface during pregnancy and may be critical to the survival of the semiallogenic fetus. A unique feature of this gene is a 13-bp deletion in the proximal promoter that renders it unresponsive to transactivation by the nuclear factor-κB (NF-κB). We previously showed that the proximal promoter of *Paan-AG*, the functional homologue of *HLA-G* in the olive baboon (*Papio anubis*), is intact. We cloned the promoters of two putative *Paan-AG* alleles (AG1 and AG2) and identified a number of regulatory elements including two κB sites. In the current study, binding and activity of the two κB elements in each putative allele were assessed by electrophoretic mobility shift and supershift assays. Functional activity was determined using luciferase reporter assays. The κB1 and κB2 elements in AG1 bound NF-κB with similar affinity. In contrast, the κB1 element of AG2 bound NF-κB with a much higher affinity than AG-1 κB1 (a 30-fold increase), whereas κB2 did not bind. Mutagenesis analysis showed that the difference in binding intensities was due to two nucleotides in the 3′ end of κB1. Similarly, failure of AG2 κB2 binding was a result of the last nucleotide in the 3′ end that differed from the consensus; mutating this nucleotide to match the consensus reestablished binding. Functional activity of the two putative alleles also differed; AG1 luciferase activity was consistently lower than that of AG2. Mutating the last two nucleotides in the 3′ end of AG1 κB1 resulted in increased luciferase activity to levels comparable to that of AG2. Overall, these results show that in vitro variations in the promoter region may influence transcription of *Paan-AG*.

Keywords Proximal promoter · Baboon · Nonhuman primate · MHC · Untranslated region

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Introduction

The human leukocyte antigen (HLA) class I genes encode glycoproteins that play critical roles in tissue graft rejection and immune responses to viruses and neoplastically transformed cells (David-Watine et al. 1990). Expression of these genes is regulated by *cis*-acting regulatory elements located in the proximal promoter, which determine their constitutive and cytokine-induced levels. These include Enhancer A elements, interferon-stimulated responsive element, and the SXY module that is shared with HLA class II genes (van den Elsen et al. 1998). The Enhancer A region contains binding sites for the transcription factor nuclear factor-κB (NF-κB), a dimeric protein that mediates cellular responses to a wide variety of stimuli including tumor necrosis factor (TNF-α), lipopolysaccharide (LPS), interleukin-1, and ultraviolet light (Ghosh et al. 1998).

NF-κB plays an integral role in many important and diverse processes including inflammatory responses, immune system development, apoptosis, learning, and bone development (Ghosh et al. 1998). The κB binding sites in the promoters of most eukaryotic genes display a remarkably loose consensus sequence, cited as $G_{-5}G_{-4}G_{-3}R_{-2}N_{-1}N_0Y_{+1}Y_{+2}C_{+3}C_{+4}$ (Ghosh et al. 1998). Crystal structures have shown that the NF-κB p50 homodimers bind specifically to 5′-$G_{-5}G_{-4}G_{-3}$ whereas NF-κB p65 homodimers interact specifically with 5′-$G_{-5}G_{-4}$. In contrast, NF-κB p50:p65 heterodimers bind to a variety of κB site sequences (Chen and Ghosh 1999; Berkowitz et al. 2002). HLA class Ia genes bind to and are responsive to transactivation by NF-κB (Gobin et al. 1998). In contrast, the class Ib genes differ. Although the HLA-E κB element is divergent and does not bind NF-κB, the HLA-F κB element displays a strong homology to that of HLA class Ia genes; it binds to, and is strongly transactivated by NF-κB. The HLA-G promoter is unique; a 13-bp deletion eliminates the 3′ end of the kB1 element, resulting in binding of only the inactive p50 subunit of NF-κB (Gobin and van den Elsen 2000).

HLA-G has generated considerable interest because of the unique characteristics that distinguish it from other class I major histocompatibility complex (MHC) genes and its potential role in modulating the maternal immune responses to the semiallogenic fetus, resulting in pregnancy success (reviewed in Hunt et al. 2005). We have been assessing the suitability of using the olive baboon (*Papio anubis*) as a model for HLA-G in vivo studies. The baboon expresses proteins encoded by *Paan-AG*, a unique *HLA-G*-like MHC class Ib gene, in the placenta (Langat and Hunt 2002; Langat et al. 2002). The two genes display remarkable similarity in the coding region and expression of the protein, suggesting they may have similar regulatory mechanisms. However, the two genes differ in the proximal promoter. Whereas the proximal promoter of *HLA-G* contains a 13-bp deletion and is unresponsive to interferon regulatory factor (IRF) and NF-κB transactivation, the *Paan-AG* proximal promoter is intact and can be transactivated by IRF (Langat et al. 2004). In the current study, we sought to identify transcription factors that bind to κB elements of the *Paan-AG* proximal promoter and assessed the effects of polymorphisms in these elements on promoter activity.

Materials and methods

Cell culture

The nonadherent baboon lymphoblast cell line 26CB-1 (American Type Culture Collection, Manassas, VA) was cultured in RPMI 1640 medium (Mediatech Herndon, VA) containing 2 mM L-glutamine and 10% (*v*/*v*) heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals®, Lawrenceville, GA). The human embryonic kidney cell line HEK293 (ATCC) was cultured in Dulbecco modified essential medium with 2 mM L-glutamine and Earle balanced salt solution, adjusted to contain 1.5 g/l sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and 10% FBS. Confluent HEK293 cells in T-75 flasks were treated with TNF-α (5 ng/ml, R&D Systems, Minneapolis, MN) for different times (5, 10, 15, 30, 60, 90, 120, 150, and 180 min). Adherent HEK293 cells were washed with 0.1 M sterile phosphate-buffered saline (PBS, pH 7.2) and treated for 5 min with a solution containing 0.25% (*w*/*v*) trypsin and 0.53 mM ethylenediamine tetraacetic acid (EDTA). Cells were harvested by centrifugation at 200×*g* for 10 min at 4°C, washed twice with cold PBS, counted, and kept on ice until ready for nuclear extraction. For luciferase assays, cells were seeded into six-well culture plates (Corning, Corning, NY) at a concentration of 3×10^5 cells per well in appropriate medium (described above) and cultured for 24 h before transfection.

Preparation of nuclear extracts

Ten million cells were resuspended in 500 μl of ice-cold hypotonic solution containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.9, 1.5 mM magnesium chloride ($MgCl_2$), 10 mM potassium chloride (KCl), 0.5 mM dithiothreitol (DTT), and 0.2 mM phenylmethylsulphonylfluoride (PMSF) and left on ice for 10 min. The cells were then transferred to a Dounce homogenizer prechilled on ice (2-ml capacity, B-pestle) and homogenized with ten strokes to disrupt the membranes. The homogenate was centrifuged at 1,000×*g* for 10 min at 4°C to recover the nuclear fraction. The supernatant was discarded, and the nuclear pellet was gently resuspended in 200 μl of low-salt buffer (20 mM HEPES pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.02 M KCl, 0.5 mM DTT, 0.2 mM PMSF) and transferred to a 1.5-ml ultracentrifuge tube on ice. Two hundred microliters of high-salt buffer (same as low-salt buffer but containing 1.2 M KCl) was added dropwise while on ice and mixed in a circular rotator for 30 min at 4°C. The nuclear extract was recovered from chromosomal DNA by ultracentrifugation at 100,000×*g* (Beckmann Coulter, Fullerton, CA) for 1 h at 4°C. The supernatant was dialyzed against 500 ml of dialysis buffer (20 mM HEPES, pH 7.9, 20% glycerol, 0.2 mM EDTA, 100 mM KCl, 0.5 mM DTT, 0.2 mM PMSF) for 12 h at 4°C. Protein concentration was determined using the BioRad Protein Assay kit (BioRad, Hercules, CA) according to manufacturer's instructions. The nuclear extracts were then aliquoted, snap-frozen in liquid nitrogen, and stored at −80°C until use.

Oligonucleotide labeling and electrophoretic mobility shift assay

Double-stranded oligonucleotides (Table 1) containing the putative NF-κB binding sites from the promoters of two putative alleles of *Paan-AG* (5′UTAG-1 and 5′UTAG-2, hereafter referred to as AG1 and AG2, respectively) and HLA-A2 (positive control) were labeled using T4 Polynucleotide kinase (Promega, Madison, WI) and [γ-^{32}P]ATP, 4,500 Ci/mmol at 10 mCi/ml (MP Biochemicals, Irvine, CA). Nuclear extracts (~5 μg protein) were incubated with 0.035 pmol of labeled probe for 20 min at room temperature in DNA/protein-binding buffer (50 mM *Tris*-HCl, pH 7.5, 250 mM NaCl, 5 mM $MgCl_2$, 20% glycerol, 2.5 mM EDTA, 2.5 mM DTT, and 0.25 mg/ml poly(dI-dC)·poly(dI-dC). Samples were loaded onto a 4% polyacrylamide gel and electrophoresis performed for 3 h at 100 V. The gel was dried for 1 h and exposed to X-ray film (Hyperfilm™ MP, Amersham Biosciences, Buckinghamshire, UK) overnight at −70°C. The films were assessed by densitometry using Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD). For competition studies, unlabeled oligonucleotides were added to the nuclear extracts 10 min before the labeled probe to confirm specificity of the binding of the labeled probe. Identity of the specific NF-κB–DNA complex was confirmed by supershift assays that involved preincubating nuclear extracts for 1 h at 4°C with polyclonal rabbit anti-p65 or goat anti-p50 NF-κB antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) before addition of the labeled oligonucleotides.

Table 1 Sequence of oligonucleotide probes used for electrophoretic mobility shift assays

Name	Sequence
Consensus[a]	$G_{-5}G_{-4}G_{-3}R_{-2}N_{-1}N_0Y_{+1}Y_{+2}C_{+3}C_{+4}$
AG1 κB1	5′-cagggttg**GGGGTTCCTA**atct-3′
AG1 κB1-m1[b]	5′-cagggttg**GGGATTCCTA**atct-3′
AG1 κB1-m2	5′-cagggttg**GGGGTTCCCC**atct-3′
AG1 κB1-m3	5′-cagggttg**GGGGTTCCCC**atct-3′
AG2-κB1	5′-caacgttg**GGGATTCCCC**atct-3′
HLA-A κB1	5′-cagccttg**GGGATTCCCC**aact-3′
HLA-A κB2	5′-cttg**GGGAGTCCCA**gcctt-3′
AG1 κB2	5′-gctg**GGGGGTCCCA**gggtt-3′
AG2 κB2	5′-actg**GGGAGGCCCA**acgtt-3′
AG2 κB2 m1	5′-actg**GGGAGGCCCC**acgtt-3′
AG2 κB2 m2	5′-actg**GGGAGCCCCA**acgtt-3′
AG2 κB2 m3	5′-actg**GGGAGTCCCA**acgtt-3′
OCT1	5′-tgtcgaatgcaaatcactagaa-3′

[a] Consensus sequence: *R* purine; *N* any base; *Y* pyrimidine

[b] The underlined bases are the sites of mutagenesis of AG1 κB1 or AG2 κB2.

Luciferase assay

The reporter constructs pGL3-5′UTAG-1 and pGL3-5′ UTAG-2 were generated by cloning a 300-bp fragment of AG1 or a 352-bp fragment of AG-2 *Paan-AG* promoter into the *Mlu*I/*Bgl*II restriction sites upstream of the firefly luciferase gene in pGL3-Basic vector (Promega, Madison, WI) as previously described (Langat et al. 2004). The positive control reporter construct pGL3-HLA-B contained a 461-bp fragment of the promoter of HLA-B*510116 gene cloned into the *Nhe*I/*Hin*dIII restriction sites upstream of the firefly luciferase gene in pGL3-Basic vector. All inserts were verified by sequencing using an ABI Prism™ DNA sequencing kit and an ABI 377 Prism™ automated sequencer (PE Applied Biosystems, Foster City, CA). Adherent HEK293 cells (90% confluent, in six-well plates) were transfected with a mixture of 4 μg pGL3 reporter plasmid DNA and 0.05 μg *Renilla* luciferase control plasmid (pRL-SV40, Promega) using Lipofectamine™ and Plus™ Reagents and manufacturer's protocols (Invitrogen, Carlsbad, CA). Luciferase activity was assessed 24–48 h after transfection using the Dual-Luciferase® Reporter Assay kit and protocols (Promega). Transfection efficiency was corrected using *Renilla* luciferase activity values.

Site-directed mutagenesis

The two *Paan-AG* alleles, AG1 and AG2, differ by three nucleotides in the κB1 binding region (Langat et al. 2004). To determine the effect of this polymorphism on promoter activity, each of the three nucleotides in AG1 κB1 was mutated in turn to resemble AG2 κB1 (Table 1) using the QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA). Activities of the mutant plasmids (AG1 m1, m2, and m3) were assessed by luciferase assay as described in the text above. Data were compared by Student *t* test analysis using SigmaStat 2.0 (Jandel Scientific, San Rafael, CA).

Results

The two *Paan-AG* κB elements differentially bind NF-κB transcription factors

The promoter region of HLA class I genes contain two putative κB sites, κB1 and κB2 (proximal and distal κB sites, respectively) in their enhancer elements. The highly conserved κB1 site in the enhancer A (GGGATTCCCC) binds homodimers and heterodimers of the NF-κB/Rel family (Gobin et al. 1998) and was used as a reference to test for binding of NF-κB to the enhancers of *Paan-AG*.

Using the *HLA-A* κB1 element as a positive control and nuclear extracts from 26CB-1 cell line, the ability of the putative κB binding sites from the *Paan-AG* alleles AG-1

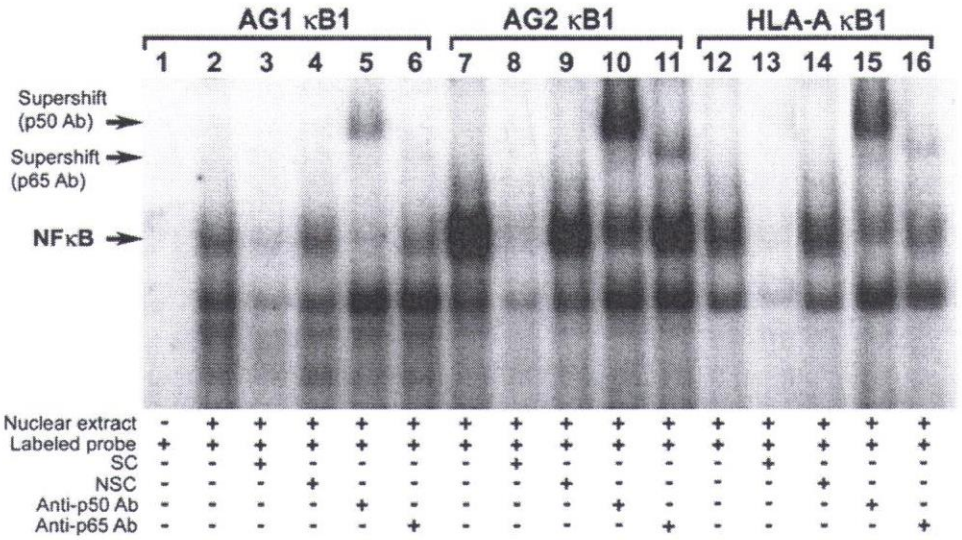

Fig. 1 NF-κB transcription factors bound to the κB site in enhancer A in the proximal promoter of *Paan-AG* gene. Electrophoretic mobility shift assay analysis of nuclear extracts from a 26CB-1 baboon lymphoblast cell line showed binding to the κB1 site of AG1 and AG2 alleles (*lanes 2* and *7*). Specificity of binding was confirmed by specific (*lanes 3* and *8*) and nonspecific (*lanes 4* and *9*) competition with unlabeled oligonucleotides. The identity of the bound complexes was confirmed by supershift analysis using anti-p50 (*lanes 5* and *10*) and anti-p65 (*lanes 6* and *11*) antibodies. The highly conserved HLA-A κB1 (Gobin et al. 1998) was used as a positive control (*lanes 12–16*). Note that there is a supershift in *lane 6*, but it is very faint. *SC*, specific competition; *NSC*, nonspecific competition; *Ab*, antibody

and AG-2 (Langat et al. 2004) to bind NF-κB transcription factors was assessed. Figure 1 shows that the proximal κB site, AG1 κB1, of both AG1 and AG2 alleles bound NF-κB (lanes 2 and 7). Specificity of binding was confirmed by competition studies using unlabeled probes (lanes 3 and 8), which successfully competed for the κB1 binding sites. Unlabeled probes that bind other elements, such as OCT1, did not bind the κB1 binding site (lanes 4 and 9). The identity of the bound complexes was determined using supershift assays with anti-p50 and anti-p65 antibodies. Both antibodies confirmed that the p50 and p65 homodimers or heterodimers were contained in the upper band (arrowhead, Lanes 5,6,10, and 11). AG-2 κB1 consistently bound NF-κB with a higher intensity than AG-1. The pattern of mobility shift and supershift was comparable to that of the positive control probe, HLA-A κB1 (lanes 12 through 15).

The binding affinity of the distal κB2 site of both *Paan-AG* alleles was also assessed and compared to that of the proximal κB1. Figure 2 shows that AG-1 κB2 specifically bound the NF-κB transcription factors (lane 5), similar to the κB1 site (lane 2) as previously observed. In contrast, AG-2 κB2 did not bind the transcription factors or bound with low affinity (lane 11).

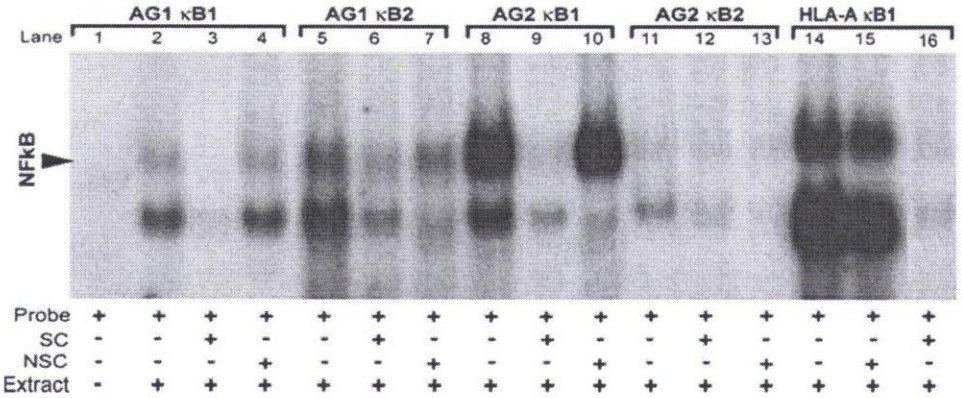

Fig. 2 Binding of NF-κB to the proximal (κB1) and distal (κB2) sites of enhancer A in the putative AG1 and AG2 putative alleles. AG1 κB1 and κB2 (*lanes 2–7*) and AG2 κB1 (*lanes 8–10*) bound to NF-κB, with AG2 κB1 consistently binding with a stronger affinity. In contrast, AG2 κB2 did not bind. HLA-A κB1 was used as a positive control. The nuclear extract was from the baboon cell line, 26CB-1. *SC*, specific competition; *NSC*, nonspecific competition

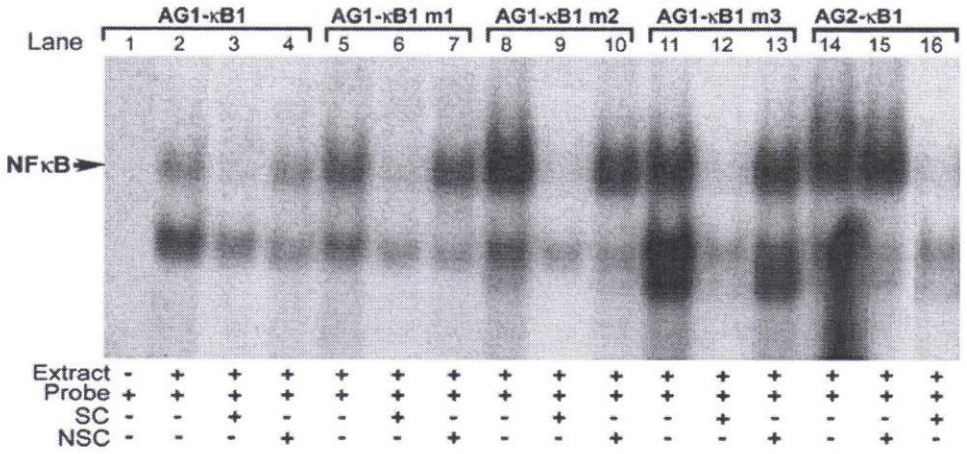

Fig. 3 The intensity of binding of κB elements to NF-κB is dependent on the sequence of the element. AG1 κB1 (*lane 2*) bound NF-κB with less affinity than AG2 κB1 (*lane 14*). The two elements differed by three nucleotides, and by sequentially replacing each of the three nucleotides of AG1 κB1 with those of AG2 κB1, the intensity of binding increased significantly (*lanes 5, 8, 11*) to levels similar to that of AG2 κB1. The nuclear extract was from the baboon cell line, 26CB-1. *SC*, specific competition; *NSC*, nonspecific competition

Allele-specific variation of the κB sites of *Paan-AG* gene and effects on binding

Comparison of the κB1 site sequences of the two putative *Paan-AG* alleles showed that they differ by three nucleotides at positions −2, +3, and +4 (locations relative to the consensus, Table 1; Ghosh et al. 1998). To confirm whether the difference in the binding affinity reported here was due to differences in the nucleotide sequences, we sequentially mutated each of the three nucleotides in AG-1 κB1 to that of AG-2 κB1 sequence (Table 1). Results showed that the binding affinity was dependent on the nucleotide sequence of the κB site. Mutating the nucleotide at position −2 (G to A, AG1 κB1 m1) increased the binding almost threefold (Fig. 3). However, mutating the last two nucleotides (positions 3 and 4, TA to CC, AG1 κB1 m2) increased the binding affinity 84-fold (lane 8, Fig. 3), superseding even that of AG2 κB1 (lane 14). Mutating the nucleotide at position 3 alone (T to C, AG1 κB1 m3) increased the binding affinity 53-fold, to the same level as that of 5′ UTAG-2 κB1 (Fig. 3, lane 11). The specificity of binding of each probe was confirmed by competition experiments using unlabeled specific and nonspecific probes.

Figure 2 shows that the AG2 κB2 element did not bind transcription factors. Comparison of the sequence with that of the consensus showed that AG2 κB2 differed by two nucleotides (Table 1). We sequentially mutated each of these nucleotides to confirm whether the sequence was critical for binding. Changing the last 'A' to a 'C' (GGGAGGCCC*A* to GGGAGGCCC*C*, AG2 κB2 m1) reestablished the consensus (Table 1) and restored binding (Fig. 4, lane 5). Similarly, changing the middle 'G' to a pyrimidine (GGGAG*G*CCCA to GGGAG*C*CCCA, AG2 κB2 m2) or GGGAG*G*CCCA to GGGAG*T*CCCA, AG2 κB2 m3) restored binding (Fig. 4, lanes 8 and 11, respectively). The latter two mutations shift the binding site to begin with the 'g' located 5′ to the consensus (Table 1).

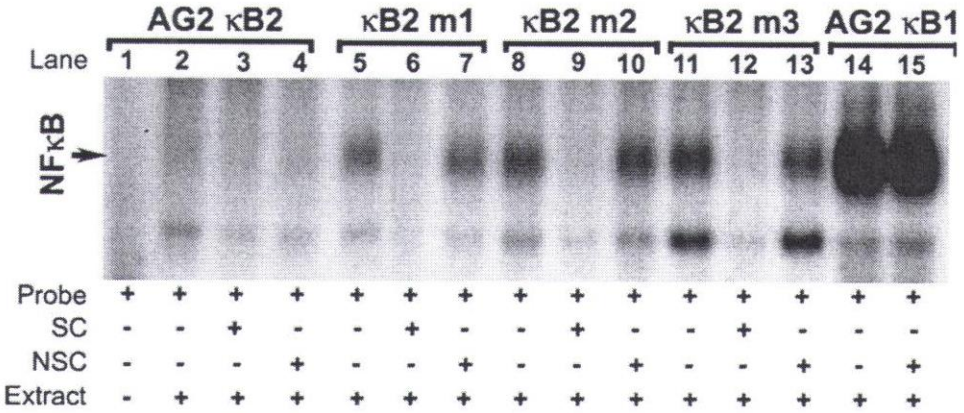

Fig. 4 The intensity of binding of κB elements to NF-κB is dependent on the sequence of the element. AG2 κB2 (*lane 2*) did not bind NF-κB (compare with AG2 κB1, *lane 14*). The two elements differed by two nucleotides, and by mutating each of the two nucleotides of AG2 κB2 with those of the consensus, the binding was restored (*lanes 5, 8, 11*). The nuclear extract was from the baboon cell line, 26CB-1. *SC*, specific competition, *NSC*, nonspecific competition

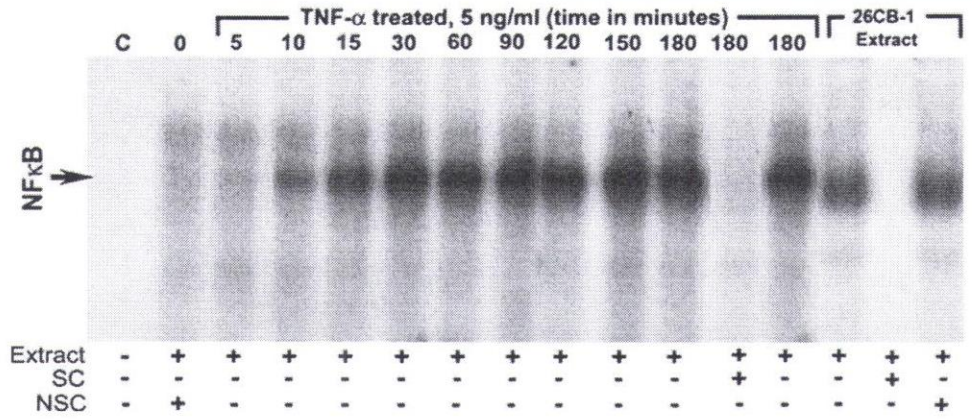

Fig. 5 The baboon enhancer A elements, AG2 κB1, bind transcription factors in the human embryonic kidney cell line, HEK293. No binding was observed in nuclear extracts obtained from untreated cells. On treatment with TNF-α, the κB elements bound to NF-κB in a dose-dependent manner. Extracts from the baboon cell line, 26CB-1 (which is constitutively active) was used as a positive control. *C*, control reaction with no nuclear extract; *SC*, specific competition; *NSC*, nonspecific competition

Paan-AG κB1 element binds NF-κB in HEK293 nuclear extracts

Nuclear extracts obtained from cultured HEK293 cells were incubated with labeled AG1 and AG2 κB1 probes. As shown in Fig. 5, no binding was observed. However, extracts from HEK293 cells treated with TNF-α for different time periods bound to *Paan-AG* κB1, with the binding intensity increasing in a time-dependent manner (Fig. 5). As previously observed above, AG2 κB1 bound to the transcription factors with a higher intensity (Fig. 5) than AG1 κB1 (results not shown).

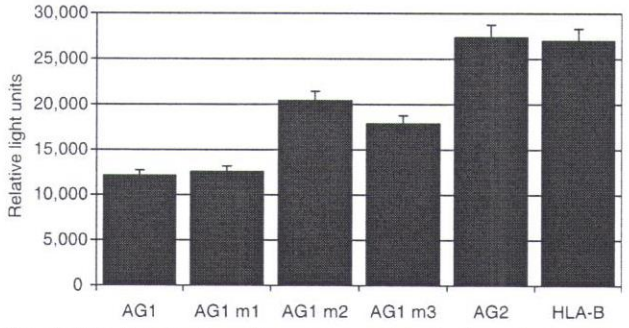

Fig. 6 NF-κB induction of promoter activity is dependent on the nucleotide sequence of the κB element. Transient transfection assays were performed using plasmids containing the proximal promoter of AG1 and AG2 *Paan-AG* putative alleles cloned upstream of the luciferase reporter gene in the pG3-Basic plasmid. Firefly luciferase activity values were normalized with *renilla* luciferase activity and expressed as ±SD of $n=3$. Luciferase activity was consistently higher in AG2 κB1-containing plasmids compared to AG1 κB1-containing plasmids. Mutagenesis of single nucleotide differences in the κB1 region (Table 1) showed that the differences in transcriptional activity were due to the nucleotide differences. m1, m2, m3; plasmids containing mutated κB1 nucleotide sequence

Nucleotide differences in the promoter influence transcription activity

The effect of NF-κB transcription factor binding on the activity of the promoter was assessed by luciferase assays. The promoter of *HLA-B*, previously shown to be transcriptionally active in transient transfection assays (Gobin et al. 1998), was used as a positive control. Transcriptional activity of the *Paan-AG* alleles differed significantly, with that of AG2 κB1 being consistently higher than that of AG1 κB1 (Fig. 6).

As indicated before, AG1 κB1 differed from AG2 κB1 by three nucleotides (Table 1). To test whether the differences in transcriptional activity was due to the nucleotide differences in the two alleles, we used site-directed mutagenesis to generate three mutant plasmids from pGL3-AG1. The mutants, containing one or two nucleotide changes in the κB1 site (substituting the nucleotides in

Table 2 Binding intensities of transcription factors to κB elements in *Paan-AG* alleles

	κB1	κB2
AG1	+[a]	+
AG2	+++	0
HLA-A	++	++
AG1 m1[b]	+	N/A
AG1 m2	+++	N/A
AG1 m3	+++	N/A
AG2 m1[c]	N/A	++
AG2 m2	N/A	++
AG2 m3	N/A	++

N/A Not applicable
[a] Level of binding intensity
[b] AG1 m1-3 are mutants of AG1 κB1.
[c] AG2 m1-3 are mutants of AG2 κB2.

AG1 with those of AG2, Table 1), were then used in transient transfections and activity assessed using luciferase assays. As shown in Fig. 6, mutating the nucleotide at position −2 (G to A, AG1 m1) did not result in a significant change in transcriptional activity. However, mutating the last two nucleotides (positions 3 and 4, TA to CC, AG1 m2) resulted in a significant increase in transcriptional activity ($p<0.05$), similar to changing the nucleotide at position 3 alone (T to C, AG1 m3). Treatment of transfected HEK293 cells with either TNF-α or LPS resulted in a significant reduction in luciferase activity (results not shown).

Discussion

This study demonstrates that polymorphisms, which differ in the two putative alleles in the promoters of *Paan-AG*, significantly influence the expression level of this gene. The promoters of two putative *Paan-AG* alleles containing the κB binding sequence (Langat et al. 2004) bound NF-κB dimers with different affinities. The level of binding of transcription factors to the κB elements in the promoter of *Paan-AG* was dependent on the nucleotide sequence of the promoter element. One or two nucleotide changes were sufficient to change the binding of transcription factors to the promoter significantly (Fig. 1). This was consistent with a previous report showing that a single nucleotide change in the κB site was sufficient to alter binding of NF-κB dimers (Leung et al. 2004).

The sequence of AG2 κB1 was identical to that of *HLA-A* (Table 1), which is highly conserved and had previously been shown to bind both homodimers and heterodimers of p50 and p65 subunits of NF-κB (Gobin et al. 1998). This may explain the similarity in transcription factor binding to the κB1 elements of AG2 and HLA-A (Fig. 1). In contrast, the proximal and distal κB sites of AG2 (κB1 and κB2) differ by three nucleotides located in positions −1, 0, and 2 (Table 1). We observed that AG2 κB1 binds NF-κB with high intensity whereas AG2 κB2 did not (Fig. 2, Table 2), suggesting that these nucleotides may be critical in binding of NF-κB transcription factors. Mutating the nucleotide at position 2 ('A' to 'C') restored the consensus and binding, although the intensity was lower than that of AG2 κB1. Similarly, mutating the nucleotide at position 0 to a pyrimidine reestablished the consensus and binding (Fig. 5). However, the latter mutation shifts the binding site to begin with the 'g' immediately 5′ of the consensus (Table 1). These results confirm the importance of the nucleotide sequence in the binding of transcription factors and in activity of the promoter.

A potential limitation of these studies is that they were performed using tumor cell lines, which, like most tumors, have altered properties and do not always mimic primary cell responses. For example, the baboon cell line 26CB-1 was constitutively active as shown by the presence of active NF-κB in nuclear extracts from untreated cells (Figs. 1, 2, 3, and 4). In contrast, the human embryonic kidney cell line HEK293 required activation with TNF-α to induce activation and translocation of NF-κB to the nucleus (Fig. 5). In both extracts, AG2 κB1 binding was consistently higher than that of AG1 κB1. It is likely that the differences in activity of these two promoters may be biologically significant in vivo, but this awaits confirmation in primary cells.

The data presented in this study demonstrate that differences in the nucleotide sequences of the promoter of *Paan-AG* significantly influence binding to, and transcriptional activity of κB elements in the promoter (summarized in Table 2). These data are similar to recent reports showing that a single nucleotide polymorphism in the promoter of *HLA-G* is associated with differences in the transcriptional activities of different *HLA-G* alleles and may be linked to sporadic miscarriages (Ober et al. 2006). Although *Paan-AG* and *HLA-G* differ significantly in the proximal promoter because of the 13-bp deletion in *HLA-G* (Solier et al. 2001; Langat et al. 2004), the two genes share many unique characteristics. These include limited polymorphism, alternative splicing of mRNA that encode proteins with truncated cytoplasmic regions, and high expression of soluble and membrane-anchored protein isoforms in the placenta (Hunt et al. 2005; Langat et al. 2002; Langat and Hunt 2002). Previous reports showed that the *HLA-G* promoter is polymorphic, and this characteristic may be related to this gene's highly regulated expression pattern (Ober et al. 2003; Tan et al. 2005). We also observed multiple nucleotide variations in the promoter of *Paan-AG* (Langat et al. 2004). Collectively, the results to date suggest that *Paan-AG* and *HLA-G* expression may be regulated via different mechanisms. The mechanisms regulating the highly restricted tissue expression of both genes remain to be elucidated.

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