Keratinocyte Responsive Element 3: Analysis of a Keratinocyte-specific Regulatory Sequence in the 230-kDa Bullous Pemphigoid Antigen Gene Promoter

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The 230-kDa bullous pemphigoid antigen gene is expressed primarily, if not exclusively, in basal keratinocytes of the epidermis. Keratinocyte responsive element 3, a *cis*-element at position −216 to −197 of the human 230-kDa bullous pemphigoid antigen gene promoter, confers tissue-specific expression to this gene (Tamai *et al*: *J Biol Chem* 270:7609–7614, 1995). In this study, we investigated the functional characteristics of keratinocyte responsive element 3 on the 230-kDa bullous pemphigoid antigen gene core promoter by transient transfections of cultured normal human keratinocytes and normal human fibroblasts, as well as of lung carcinoma (A549), osteosarcoma (OST), and gastric adenocarcinoma (GT3TKB) cell lines. A 230-kDa bullous pemphigoid antigen gene core promoter/luciferase reporter gene plasmid construct, pBPL, was modified to develop a series of constructs (pKBPL–p4KBPL), which have insertions of one, two, three, or four tandem repeats of keratinocyte responsive element 3, and these plasmids were used in transient transfections of the cultured cells. The promoter activities of pKBPL–p4KBPL constructs, relative to pBPL, in normal human keratinocytes were 7.6-, 15.5-, 4.6-, and 2.7-fold higher, respectively, whereas no upregulatory effect by keratinocyte responsive element 3 insertion was observed in other cell lines tested. prKBPL, a plasmid constructed with keratinocyte responsive element 3 in reverse orientation, showed essentially no activity in normal human keratinocytes. Insertion of a random 20 bp sequence between keratinocyte responsive element 3 and the 230-kDa bullous pemphigoid antigen gene core promoter resulted in about 40% reduction of luciferase activity in normal human keratinocytes. These data suggest that keratinocyte responsive element 3 functions as a position-, copy number-, and orientation-dependent *cis*-element contributing to tissue-specific regulation of the 230-kDa bullous pemphigoid antigen gene. *Key words: Bullous pemphigoid antigens/keratinocyte gene expression/transcriptional regulation. J Invest Dermatol 120:308–312, 2003*

Bullous pemphigoid (BP) is a cutaneous blistering disease characterized by the presence of circulating IgG autoantibodies in the patients' sera (Lever, 1965; Stanley *et al*, 1981). Western blot analyses of BP patients' sera have recognized at least two distinct proteins that are associated with epidermal keratinocytes, so-called bullous pemphigoid antigens (BPAGS), which serve as autoantigens in the disease. The major antigen recognized in most patients' sera is a 230-kDa protein, designated as BPAG1, whereas another protein, BPAG2, recognized in relatively few patients' sera, has a molecular weight of 180-kDa (Mueller *et al*, 1989; Meyer *et al*, 1990; Sawamura *et al*, 1992). Immunoelectron microscopic studies have indicated that these two autoantigens are associated with hemidesmosomes, attachment complexes spanning from the intracellular milieu of the basal keratinocytes to the underlying lamina lucida (Westgate *et al*, 1985; Mutasim *et al*, 1985). cDNA cloning and chromosomal mapping of the BP antigen genes have clearly demonstrated that BPAG1 and BPAG2 are distinct gene products (Sawamura *et al*, 1992). Specifically, cloning of the BPAG1 cDNA has demonstrated that the 230-kDa protein is a noncollagenous component of the intracellular hemidesmosomal plaque (Sawamura *et al*, 1991a,b; Tanaka *et al*, 1991). In contrast, the 180-kDa BP antigen is a transmembrane collagenous protein (Giudice *et al*, 1991, 1992; Hopkinson *et al*, 1992; Li *et al*, 1992), designated as type XVII collagen (Li *et al*, 1993). In addition to cDNA cloning, the entire gene structure and the intron–exon organization of the human BPAG1 gene have been delineated (Tamai *et al*, 1993). This gene has been shown to consist of 22 distinct exons spanning ~20 kb of the genomic DNA in the short arm of human chromosome 6 (Sawamura *et al*, 1990; Tamai *et al*, 1993).

The expression of the BPAG1 gene in the skin, as determined at the mRNA level, has been shown to be limited to keratinocytes with proliferative, basal keratinocyte-like phenotype (Arnemann *et al*, 1993). The specificity of the expression of this gene has also been demonstrated by transient transfection studies utilizing the BPAG1 gene promoter/chloramphenicol acetyltransferase reporter gene constructs in various cell types (Tamai *et al*, 1993). Subsequently, a specific *cis*-regulatory element, designated as keratinocyte responsive element (KRE) 2, has been identified

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Abbreviations: BPAG1, bullous pemphigoid antigen 1; KRE3, keratinocyte responsive element 3; NHK, normal human keratinocytes; NHF, normal human fibroblasts.

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at position −1786 to −1778 (upstream from the transcription initiation site) within the BPAG1 promoter (Tamai *et al*, 1993, 1994). KRE2 was shown to have AP2 binding activity, whereas keratinocyte nuclear extract also had another KRE2-binding protein, designated as a KTP-1 (Tamai *et al*, 1994). Elimination of KRE2 sequence from the BPAG1 promoter resulted in approximately 60% reduction in its activity in keratinocytes (Tamai *et al*, 1993). Subsequent studies revealed a novel *cis*-element, KRE3, at position −216 to −197 of the human BPAG1 gene promoter (Tamai *et al*, 1995a). A BPAG1 promoter/chloramphenicol acetyltransferase construct containing KRE3 had up to ~50-fold higher expression than a similar construct devoid of this sequence when tested in transient transfections of cultured human keratinocytes. Thus, KRE3 was postulated to confer keratinocyte-specific expression to the BPAG1 gene (Tamai *et al*, 1995a).

In this study, we examined the functional characteristics of KRE3 to determine the specificity of its position with regard to the BPAG1 core promoter, its copy number requirements, and orientation responsible for conferring tissue-specific regulation of the expression of the BPAG1 gene.

MATERIALS AND METHODS

Plasmid constructs The human BPAG1 core promoter region, extending from position −1 to −196 counting upstream from the transcription initiation site was inserted in front of the luciferase gene of a plasmid pGEM-luc (Promega, Madison, WI), designated as pBPL construct, being devoid of the KRE3 element (5′-GTGCAAATATTT-GGCATATC-3′). As a control, the herpes simplex virus thymidine kinase (TK) promoter, position −1 to −722, was also inserted to pGEM-luc, a construct designated pTKL. Plasmids pKBPL, p2KBPL, p3KBPL, and p4KBPL were constructed by insertion of one, two, three, or four tandem repeats of the KRE3 sequence, respectively, at position −196 of the BPAG1 promoter in pBPL plasmid. Similarly, pKTKL, p2KTKL, and p3KTKL were constructed by insertion of one, two, or three KRE3 repeats in front of the TK promoter. pKins20BPL was constructed by insertion of a random 20 bp sequence at −196 of the BPAG1 promoter in pKBPL. Reverse KRE3 sequence (5′-CTATACGGTTTATAAACGTG-3′) was inserted at −196 of the BPAG1 promoter in pBPL, and this construct was referred as prKBPL. All constructs were sequenced in both directions to confirm their nucleotide sequence.

Cell cultures Normal human epidermal keratinocytes (NHK), which were obtained from Clonetics (San Diego, CA), were maintained in serum-free, low calcium (0.15 mM) keratinocyte growth medium supplemented with epidermal growth factor, hydrocortisone, insulin, and bovine pituitary extract (Clonetics). The cell cultures were treated by trypsinization and studied at passage 3. Normal human fibroblasts (NHF) were established from human biopsy specimens, and the human lung carcinoma cell line A549, the human osteosarcoma cell line OST, and the human gastric adenocarcinoma cell line GT3TKB were obtained from RIKEN Cell Bank (Tsukuba, Japan). These cultures were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 μg streptomycin per ml, and 50 units penicillin per ml.

Transient cell transfections The series of plasmid constructs described above were used for transient transfection studies in NHK, NHF, A549, OST, and GT3TKB cultures. These cells were also transfected with a cytomegalovirus promoter/luciferase reporter gene plasmid construct (pCMVL) and the luciferase reporter gene plasmid (pGEM-luc), which were used as positive and negative controls, respectively, of transfection efficiencies. In each experiment, cultured cells were transfected in 60 mm diameter dish when 70–80% confluent. All transfections were performed with commercial kit (DOTAP, Roche Diagnostics, Mannheim, Germany). After 24 h of transfection, the cell cultures were placed in fresh culture medium and grown for an additional 24 h prior to harvesting.

Luciferase assay Cultured cells were washed twice with phosphate-buffered saline and scraped into 250 μl of cell culture lysis reagent LCβ/PGC-51 (Toyo Ink, Tokyo, Japan). Insoluble protein was removed by a 2 min centrifugation at 15,000 g and the supernatant was immediately assayed for luciferase activity using commercial kit (Picagene, Toyo Ink). In the assay, 20 μl of cell extract and 100 μl of luciferin mixture were reacted for 5 s and light output was monitored over the next 10 s. All assays were performed in triplicate, and each experiment was repeated a minimum of three times. Each luciferase activity value was corrected for protein amount in each sample. The values were expressed as mean ± SD, and the statistical significance of differences between treatment groups was calculated by Student's two-tailed t-test.

RESULTS

Enhancement of BPAG1 promoter activity by KR3 is copy number dependent Previous studies have demonstrated that the BPAG1 gene is specifically expressed in epidermal keratinocytes (Stanley, 1989; Tamai *et al*, 1993), and KRE3 was suggested to confer tissue-specific expression to the BPAG1 gene (Tamai *et al*, 1995a). In this study, we examined the KRE3 functional characteristics, first by inserting various numbers of KRE3 in front of the human BPAG1 core promoter. In transient transfection studies in NHK, the relative promoter activities of pKBPL, p2KBPL, p3KBPL, and p4KBPL were 7.6-, 15.5-, 4.6-, and 2.7-fold higher, respectively, in comparison with pBPL (**Fig 1**). The highest promoter activity was obtained by insertion of two tandem repeats of KRE3. Addition of further repeats of KRE3, however, resulted in lower promoter activities as compared with one or two repeats, suggesting the importance of relative position of KRE3 elements toward the core promoter of BPAG1.

Sense orientation of KRE3 is critical for the enhancement of BPAG1 promoter activity in NHK To investigate whether the orientation of KRE3 has an effect on its *cis*-regulatory activity, prKBPL containing the KRE3 sequence in reverse orientation in front of the BPAG1 core promoter was constructed, transfected in NHK, and the promoter activity was compared with the activities of pBPL and pKBPL. As shown in **Fig 2**, prKBPL resulted in essentially no luciferase activity, which was even less than that noted with pBPL. In the same experiment

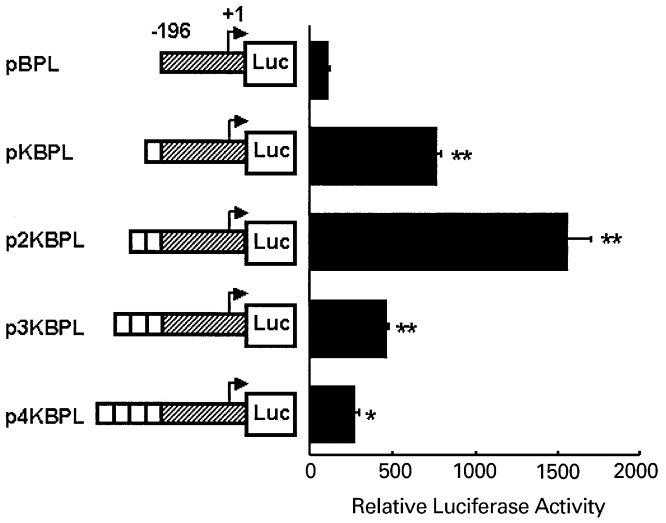

Figure 1. Effect of KRE3 on the BPAG1 core promoter activity in cultured NHK. The plasmid pBPL was constructed by insertion of the BPAG1 core promoter ▨ at the 5′ end of the luciferase gene (LUC) in the plasmid pGEM-luc. Plasmids pKBPL, p2KBPL, p3KBPL, and p4KBPL contain one, two, three, or four tandem repeats of KRE3 sequence □, respectively, at position −196 upstream from the transcription initiation site ┌► of the BPAG1 promoter in the pBPL plasmid. These constructs were transiently transfected to cultured NHK, and the promoter activities of each construct were expressed as luciferase activity ▬. The relative luciferase activity with KRE3 containing constructs was expressed in relation to pBPL which was set at 100. The values are expressed as mean ± SD from three parallel samples in three to four separate experiments, after correction of the activity for the amount of protein in the same cell extracts. The significance of differences, in relation to pBPL construct, is indicated by asterisks ($^{*}p < 0.01$, $^{**}p < 0.001$).

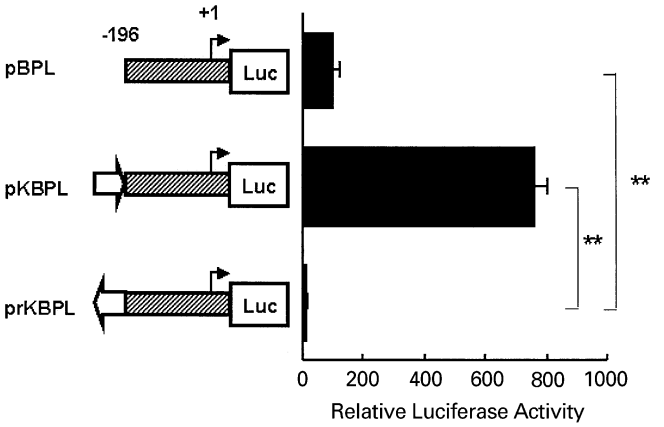

Figure 2. Effect of KRE3 orientation on the BPAG1 promoter activity in cultured NHK. The plasmid prKBPL was constructed by insertion of KRE3 reverse sequence (5′-CTATACGGTTTATAAACGTG-3′) ⇦ at the 5′ end of the BPAG1 core promoter in pBPL, and subjected to transient transfection to cultured NHK together with pBPL and pKBPL; the latter construct contains the KRE3 sequence in sense orientation. Note that prKBPL has very low promoter activity in NHK. The values are expressed as in **Fig 1**. The significance of differences is indicated by an asterisk (**$p < 0.001$).

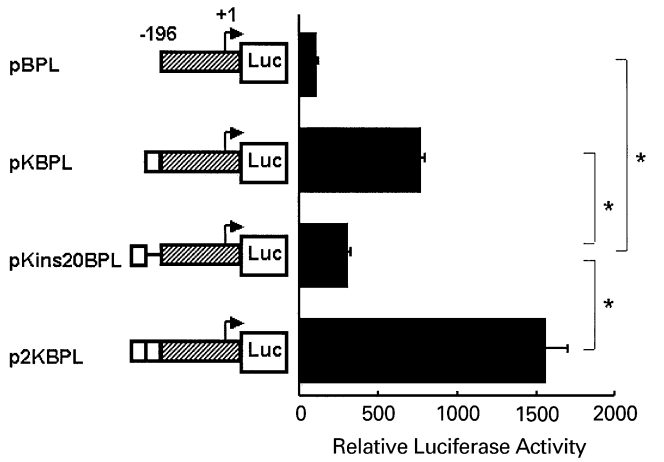

Figure 3. The positional effect of KRE3 on the BPAG1 promoter activity in cultured NHK. The plasmid pKins20BPL was constructed by insertion of a random 20 bp sequence ▬ between the KRE3 and the core promoter of BPAG1 in pKBPL. The promoter activity in cultured NHK was compared with those noted with pBPL, pKBPL, and p2KBPL, as determined by luciferase assay. About 60% lower promoter activity was obtained by pKins20BPL as compared with the activity of pKBPL. Significance of differences is indicated by an asterisk (*$p < 0.01$).

pKBPL resulted in ~7-fold enhancement of luciferase activity in comparison with pBPL. These data suggest that the spatial orientation of KRE3 is crucial for optimal transcriptional regulation on the BPAG1 promoter.

The relative position of KRE3 in relation to BPAG1 core promoter influences the activity in NHK To determine whether the relative position of KRE3 with respect to the BPAG1 core promoter has an effect on its activity, the pKins20BPL construct, which has an insertion of a random 20 bp sequence between the KRE3 sequence and the BPAG1 core promoter in pKBPL was tested in NHK cultures. Insertion of the 20 bp sequence in this construct places the KRE3 sequence to correspond to the upstream KRE3 repeat in p2KBPL. The construct pKins20BPL was then compared with pBPL, pKBPL, and p2KBPL for the promoter activity by luciferase assay. As shown in **Fig 3**, promoter activity of pKins20BPL was

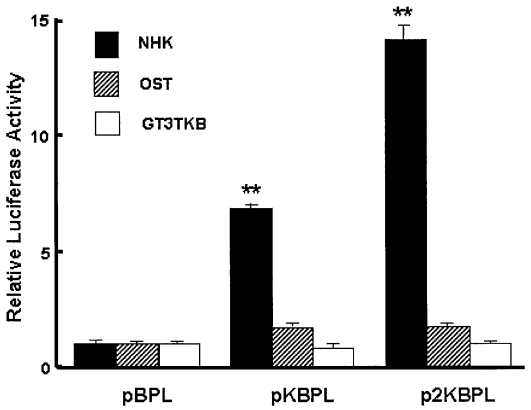

Figure 4. Cell-type specific effect of KRE3 on the BPAG1 core promoter activity in various cell lines. The plasmids pBPL, pKBPL, and p2KBPL were transiently transfected in NHK, NHF, A549, OST, and GT3TKB cell cultures, and the role of KRE3 was evaluated by luciferase assay in each cell type (see **Table I**). The values are expressed as the relative luciferase activity of pKBPL and p2KBPL, in relation to pBPL (set as 1.0), in NHK, OST, and GT3TKB, in which significant luciferase activities of the BPAG1 promoter plasmid constructs were obtained. No detectable activity was noted in NHF and A549 cells (see **Table I**). Note that KRE3 dramatically increased the BPAG1 promoter activities in NHK, but not in OST and GT3TKB cultures. Significant difference is indicated by asterisks (**$p < 0.001$).

approximately 60% lower than that of pKBPL, although it was still higher than that of pBPL. These data indicate that the relative position of KRE3 relative to the core promoter is important for its optimal activity.

The KRE3 element displays cell type specific regulatory effects Previous studies have suggested that KRE3 has a transcriptional regulatory function in the BPAG1 promoter in NHK, but not in NHF (Tamai *et al*, 1995a). To explore further the cell type specificity of the KRE3 function, the plasmids pBPL, pKBPL, and p2KBPL were transiently transfected in NHK, NHF, A549, OST, and GT3TKB cell lines, and the impact of the presence of KRE3 was evaluated by luciferase assay. The pCMVL plasmid was also transfected in these cell lines to verify transfection efficiencies. The relative promoter activities of pKBPL and p2KBPL, in comparison with pBPL, were significantly higher in NHK, whereas in OST and GT3TKB cell lines KRE3 had minimal effects on the BPAG1 promoter activity (**Fig 4**). Furthermore, the BPAG1 promoter activities were not detectable in NHF and A549 (**Table I**). These data clearly suggest that KRE3 functions as a tissue-specific transcriptional regulatory sequence in the BPAG1 gene.

Effects of KRE3 on heterologous TK promoter activity in NHK To determine whether the KRE3 element can influence heterologous promoter activity, we tested the effect of KRE3 insertion on the herpes simplex virus-TK promoter. As shown in **Fig 5**, one or two KRE3 insertions upstream to the TK in the pKTKL and p2KTKL promoter constructs did not confer any enhancement on the TK promoter activity in luciferase assay, whereas p3KTKL, containing a three KRE3 repeats insertion, resulted in a strong suppression of the TK promoter activity. This study suggested that KRE3 could have an effect on the heterologous promoter activity, but in a different manner, as compared with those effects observed in the BPAG1 promoter. We cannot, however, rigorously exclude the possibility that the different position of KRE3 in pKBPL and in pKTKL constructs (–196 and –722 with respect to transcription initiation sites) may explain the different results with these two constructs.

Table I. Expression of the BPAG1 Core Promoter Constructs in Various Cell Cultures, and Cell-Type Specific Effects of KRE3

Cell cultures:	NHK	NHF	A549	OST	GT3TKB
Luciferase Activity (arbitrary chemiluminescence units)*					
pBPL	$(3.6 \pm 0.7) \times 10^2$	ND	ND	$(2.1 \pm 0.3) \times 10^2$	$(1.8 \pm 0.3) \times 10^2$
pKBPL	$(2.5 \pm 0.2) \times 10^3$	ND	ND	$(3.5 \pm 0.7) \times 10^2$	$(1.4 \pm 0.8) \times 10^2$
p2KBPL	$(5.1 \pm 2.4) \times 10^3$	ND	ND	$(3.5 \pm 0.7) \times 10^2$	$(1.8 \pm 0.2) \times 10^2$
pCMVL	$(7.4 \pm 1.6) \times 10^6$	$(2.6 \pm 0.3) \times 10^5$	$(3.8 \pm 0.4) \times 10^5$	$(1.1 \pm 0.2) \times 10^5$	$(1.1 \pm 0.1) \times 10^5$

*The values are expressed as mean ± SD of triplicate cultures transfected in parallel.
ND: Not detected

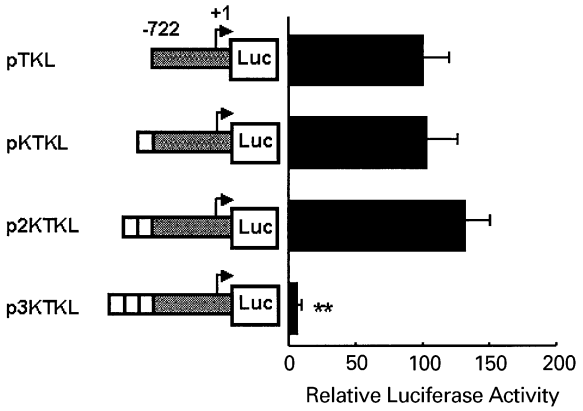

Figure 5. Effect of KRE3 on the TK promoter activity in cultured NHK. The plasmid pTKL has the TK promoter in front of the luciferase gene in the plasmid pGEM-luc. Plasmids pKTKL, p2KTKL, and p3KTKL were constructed by insertion of one, two, or three tandem repeats of KRE3 sequence at position –722 from the transcription initiation site of the TK promoter in the pTKL plasmid. These constructs were transfected to cultured NHK, and the promoter activity was determined by luciferase assay. Note that one or two KRE3 repeat insertions did not significantly affect the TK promoter activity, but p3KTKL, containing three KRE3 repeats, showed strong suppression of TK promoter activity in NHK. Significance of differences is indicated by asterisks (**$p < 0.001$).

DISCUSSION

BPAG1 is an intracellular component of hemidesmosomes, attachment complexes anchoring epidermal basal keratinocytes to the underlying basement membrane. Expression of the BPAG1 gene is restricted to stratified squamous epithelia, including epidermal keratinocytes, and its expression is regulated, at least in part, at the level of transcription. Previous studies on the BPAG1 gene promoter have reported the existence of several *cis*-regulatory elements controlling tissue-specific expression, such as KRE2 and KRE3 (Tamai *et al*, 1994, 1995a,b). Subsequent studies suggested that KRE3, a 20 bp *cis*-regulatory element extending from position –216 to –197 of the BPAG1 promoter, is necessary for epidermal-specific expression of this gene.

In this study, the functional characteristics of KRE3 were examined, and its copy number, relative position, and spatial orientation were shown to affect its activity as a tissue-specific regulatory element. Those functions were specific to epidermal keratinocytes among the cell lines so far examined, and different KRE3 activities were observed on a heterologous promoter, as exemplified by the TK gene promoter, which KRE3 can down-regulate if inserted in the form of three tandem repeats. In this context, it is of interest that KRE3 when present in one or two copies had minimal effect on TK promoter, whereas addition of the third copy in p3KTKL markedly reduced the promoter activity. A reduction in the BPAG1 core promoter activity was also noted when the third and fourth copy of KRE3 were added to the constructs p3KBPL and p4KBPL, respectively. These observations further emphasize the position-dependent nature of the effect of KRE3 on the promoter activity.

Characteristic palindromic sequence, 5′-CAAATATTTG-3′, was identified embedded within the KRE3 element (Tamai *et al*, 1995a), and palindromic structures are generally observed in *cis*-regulatory elements such as cognate AP1 and AP2 sequences. It should be noted, however, that this palindromic structure is surrounded by nonpalindromic sequences in the 20 bp KRE3 sequence inserted in front of the BPAG1 core promoter. The presence of nonpalindromic sequences may explain the orientation-dependent effect of KRE3, as it in anti-sense orientation elicited no enhancement of the BPAG1 promoter activity. This possibility is emphasized by previous demonstrations that 2 bp nucleotide substitutions both within the palindromic core as well flanking nonpalindromic sequences result in reduced promoter activity of BPAG1 (Tamai *et al*, 1995a). Previous studies indicated the existence of specific KRE3 binding nuclear proteins in keratinocytes, but not in fibroblasts (Tamai *et al*, 1995a), suggesting that there is a keratinocyte-specific, KRE3-dependent transcriptional regulatory system conferring tissue-specific expression to the BPAG1 gene. Other studies on the mouse BPAG1 promoter have defined an active region for tissue specific expression of the gene to reside between –525 and –213 of the 5′ flanking DNA (Sawamura *et al*, 1994), and this region also contains a KRE3-like sequence between –283 and –264. Recent studies have also demonstrated that homeodomain proteins, *Engrailed* and *Antennapedia*, upregulate transcription of the BPAG1 gene through their DNA binding activities of homeodomain regions, EnHD and AntpHD, respectively (Mainguy *et al*, 1999). EnHD and AntpHD, however, were shown to recognize sequences clearly different from KRE3, suggesting that tissue specificity of BPAG1 is regulated, at least in part, by nuclear proteins that are different in *Engrailed* and *Antennapedia*.

Dissection of the regulatory elements responsible for expression of various epidermal genes has revealed distinct *cis*-regulatory regions in different genes with the capacity to switch the genes on or off (Eckert *et al*, 1997). For example, recent studies on the human involucrin gene have identified the distal regulatory region as crucial for epidermal expression of the corresponding gene and shown it to reside between –2473 and –2088 in the 5′ flanking DNA (Welter *et al*, 1995). Further studies indicated involvement of Sp1 and AP1 in the distal regulatory region system, and AP1 was shown to function as the on/off switch for the gene expression (Banks *et al*, 1998). Other studies on the human SPRR3 gene promoter have shown the existence of functional AP1, Ets, and ATF/CRE binding sites in the core promoter region and have suggested cooperative regulation of these elements for tissue-specific transcription of the gene (Fischer *et al*, 1999). Most of the transcriptional systems involved in the epidermal gene regulation are shared with other tissues for their specific gene regulation, suggesting that the patterns of combination of various transcriptional factors might define tissue-specific expression of each gene. In the BPAG1 promoter region, putative CCAAT and Sp1 motifs were identified just downstream of the KRE3 sequence (Tamai *et al*, 1995a). The observations on the position- and orientation-dependent effects of KRE3 on BPAG1 core promoter activity might suggest cooperation with other *cis*-regulatory elements, including CCAAT and Sp1 motifs, for epidermal-specific expression of the gene.

In summary, we have defined several functional characteristics of KRE3, an upregulatory *cis*-element in the BPAG1 promoter. Information on the KRE3 function increases our understanding of the keratinocyte-specific expression of genes and also provides a potential pharmacologic perspective to enhance the expression of genes in the epidermis.

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