Differentiation-specific Expression of Human Keratin 1 Is Mediated by a Composite AP-1/Steroid Hormone Element*

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Human keratin 1 (HK1) expression is associated with the loss of mitotic activity in epidermal keratinocytes and restricted to an intermediate stage of terminal differentiation. Recently, the control elements that mediate this differentiation-specific expression were identified (Huff, C. A., Yuspa, S. H., and Rosenthal, D. (1993) J. Biol. Chem. 268, 377-384). We now report the characterization of one of these elements. Footprint analysis on a 249-base pair fragment containing the calcium responsive element (CaRE) revealed two adjacent protected regions. The 5' most footprint contains an AP-1 consensus sequence while the 3' footprint encodes two inverted repeats of the canonical hormone response recognition sequence. Deletion of the AP-1-protected region abolished the calcium response in a reporter construct. Calcium activation of the reporter construct containing the intact CaRE was unaffected by the addition of thyroid hormone or estrogen. However, vitamin D₃ was able to suppress calcium induction by the CaRE, and this suppression could be abrogated by the coaddition of retinoic acid. These studies show that AP-1 factors bind to the 5' element to mediate the calcium response while members of the steroid hormone receptor superfamily interact with the 3' element to modulate the calcium response.

The mammalian epidermis is composed of four histologically defined layers each of which represents a distinct stage of differentiation of the epidermal keratinocyte. Keratinocytes are the major cell type of the epidermis and arise from stem cells in the basal layer. Upon commitment to differentiation they lose their proliferative potential and migrate to the spinous layer. With further maturation they enter the granular layer and finally terminate as cornified squames in the stratum corneum before being sloughed into the environment. The degree of differentiation can also be defined biochemically by the expression of marker proteins that characterize each stage. For instance, basal keratinocytes express keratins K5 and K14. Once they enter the differentiation pathway to become spinous layer cells, they down-regulate the genes for K5 and K14 and up-regulate the genes for the differentiation-specific keratins K1 and K10. The expression of K1 precedes that of K10 and is one of the earliest events in keratinocyte differentiation. K1 can be observed in the occasional basal cell that has already ceased mitotic activity and is about to migrate into the spinous layer. Transcription of K1 and K10 is restricted to spinous layer cells, and when these mature into granular layer cells, the genes for K1 and K10 are down-regulated and other genes, notably loricrin and filaggrin, are induced. The regulation of epidermal differentiation is not fully understood, but it is known that calcium, retinoic acid, and vitamin D₃ can all act as modulators of the process.

Both in vitro and in vivo studies have implicated calcium as a major modulator of epidermal differentiation. A calcium gradient has been identified in the epidermis with the basal and spinous layers having calcium levels much lower than that observed in serum and rising to much higher than serum levels in the upper granular layer and stratum corneum (12, 13). In vitro studies have shown that keratinocytes maintain their proliferative capacity when calcium levels of the culture medium are kept below 0.1 mM and that differentiation ensues with calcium levels of 0.1 mM or higher (14). Moreover, differentiation-specific genes including keratins K1 and K10 can be induced in cultured keratinocytes by raising the level of calcium in the medium (15, 16).

In contrast, retinoic acid appears to suppress epidermal differentiation and indeed, promotes an undifferentiated phenotype in keratinocytes in culture (17). Expression of the differentiation-specific markers are also suppressed, including K1 and K10 (15, 18), loricrin (19), and filaggrin (20). In addition, a retinoic acid gradient may also exist in the epidermis, with high levels in the basal layer declining to much lower levels in the differentiated layers (21).

Another regulator of epidermal differentiation is the active metabolite of vitamin D₃, 1,25-dihydroxyvitamin D₃. It has been shown that vitamin D₃ is a potent inhibitor of proliferation (22) and, moreover, will promote the differentiation of spinous and granular cells into the corneocytes of the stratum corneum (23, 24). Vitamin D₃ is produced by keratinocytes (25), and autoradiographic studies suggest a concentration of 1,25-dihydroxyvitamin D₃ in the suprabasal layers of the epidermis (26). Until now, a direct role for vitamin D₃ on epidermal keratin gene expression has not been reported (27).

Little is known about the transcription factors that regulate the differentiation-specific genes of the epidermis. The best studied example is the HK1 gene. A 10.8-kilobase (kb) transgene, containing nucleotides -1246 to +9455 from HK1, was correctly regulated with respect to tissue and developmental specificity in transgenic mice (28-30). Furthermore, the expression of HK1 in keratinocytes cultured from these mice could be induced by the addition of calcium to the medium (30). These studies suggested that the transgene encodes all the cis-acting regulatory elements necessary to mediate the calcium re-

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‡‡ The abbreviations used are: K, keratin; HK1, human keratin 1; hp, base pairs; CaRE, calcium responsive element; kb, kilobases(s); CAT, chloramphenicol acetyl transferase; FP(A), footprint (A); FP(B), footprint (B); SV40, simian virus 40; TPA, 12-O-tetradecanoylphorbol-13-acetate.

J. Rothnagel, unpublished results.
response. Recently, an array of regulatory elements that respond to increased calcium levels in vitro were identified and found to reside within a 4.4-kb fragment, 3' of the HK1 gene (31). We have recently defined the sequences that mediate the calcium response to a 249-bp fragment that lies 7.9 kb downstream of the HK1 promoter (32).

We now report the detailed analysis of the 249-bp fragment that encodes the calcium response element (CaRE). Footprint- 
ing and mobility shift assays have identified adjacent cis-ele-
ments. One of these encodes an AP-1 element to which all of the calcium response can be attributed. The adjacent region en-
codes a hormone-responsive element through which both vita-
min D₃ and retinoic acid are able to modulate the calcium response.

MATERIALS AND METHODS

Plasmids and Constructs—The plasmid pHIVLTR.CAT (33) was used as a control. Details on the generation of the 249-bp fragment that encodes the CaRE have been given elsewhere (32). Briefly, the 249-bp (+7820 to +8069) fragment was generated by polymerase chain reaction (34) to delete the HK1 transcription) fragment was generated by polymerase chain reaction and cloned using BglII and BamHI restriction sites into the 249-bp fragment was subsequently self-ligated into four tandem repeats and inserted into the BHIII site of pAIOCATz in an antisense orientation relative to the CAT (chloramphenicol acetyltransferase) gene. This construct is referred to as p249P in Rothnagel et al. (32) and in this paper as CaRE.CAT. The constructs, CaREΔA.CAT and CaREΔB.CAT, were similarly constructed from CaREΔA and CaREΔB. These versions of the 249-bp fragment were created using polymerase chain reaction (34) to delete the entire protected region defined by footprint analysis. CaREΔA is lacking nucleotides +7924 to +7928 and CaREΔB is lacking nucleotides +7895 to +7921.

Cell Culture and Transfection—Primary murine keratinocytes were cultured and transfected as described in Harper et al. (35). Five μg of plasmid DNA plus 25 pg of carrier DNA were transfected by calcium phosphate precipitation. Cells were harvested after 48 h in these media and assayed for CAT activity (36).

The CAT assays were normalized by protein content and activity visualized by autoradiography after separation by thin layer chromatography. These experiments were repeated at least three times.

Nuclear Extracts—Nuclear extracts were made from primary murine keratinocyte cultures and prepared essentially as described by Dignam et al. (37). The protein concentration of each extract was determined by the Bradford assay (38).

Band Shift Assay—Purified fragments of either the intact CaRE, CaREΔA, or CaREΔB were end-labeled at the BglII site with DNA polymerase Klenow fragment and [32P]dATP (3,000 Ci/mmol). Each [32P]-labeled DNA fragment (10–20 fmol, 10⁶ counts/min) was incubated with 2–4 μg of nuclei protein extract and 3 μg of nonisotopic DNA poly(dI-dC) for 15 min at room temperature in 20 μl of binding buffer. The gels were dried and analyzed by autoradiography.

DNase I Footprinting—End-labeled probes for footprinting were prepared as described above. Each labeled DNA probe was incubated with 2–8 μg of nuclear protein extract or 1 footprinting unit of purified Jun–Jun (Promega), 20 ng of poly(dI-dC), and binding buffer (see above) to a total reaction volume of 20 μl. After 20 min of incubation at room temperature, the reaction was terminated by addition of 1 μl of stop solution containing 1 μg of pBR322 plasmid DNA, 15 μM EDTA, 0.15% SDS, and 1.5 μg of proteinase K. Samples were analyzed as described by Pastorici et al. (39).

Oligonucleotides—All oligonucleotides are double-stranded (only one strand is shown). AP-1 (Promega), 5'-CCGTTGATGAGTCAGCCGA-3'; NS (NON-SPECIFIC), 5'-GGGCTGGGCTCTAGAGGAAGCCGG-3'. The following sequences are from Umesono et al. (40): VDRE (DR5), 5'-AGCCCTACGAGGCTCAGAGGCTCAGAGGCT-3'; RARE (DR5), 5'-AGCCCTACGAGGCTCAGAGGCTCAGAGGCTCAGAGGCT-3'.

RESULTS

Identification of Nuclear Protein-DNA Interactions within the CaRE of HK1—Sequences within a 249-bp fragment located between +7820 to +8069 relative to the transcription start site of HK1 have been shown to direct transcription of both heterologous and homologous promoters in a cell type-specific manner in response to increased calcium levels (31, 32). To define the sequences that are important for CaRE function, we first identified factor binding sites within the 249-bp fragment. The CaRE-bearing fragment was asymmetrically end-labeled and incubated with a keratinocyte nuclear extract in the presence of DNase I. Two protected regions were predominant (Fig. 1).

We designated the 5' most footprints as FP(A) and the adjacent footprint as FP(B). The protected region of FP(A) spans nucle-
otides +7895 to +7921 and contains within it an AP-1 consensus sequence (TGATTCA) (41, 42). An examination of the sequences protected in FP(B), which spans nucleotides +7924 to +7948, revealed an inverted palindromic sequence. This region contains two half-sites of the canonical hormone response element, (A/G)GGTCA (43), separated by three nucleotides.

To confirm that binding to FP(A) is due to nuclear factor interaction with the AP-1 site, a 25-bp double-stranded oligonucleotide, containing the AP-1 consensus sequence, was added to the binding reaction. Binding to FP(A) was almost completely abolished by the addition of the competing oligonucleotide (Fig. 1, lanes 4–6) whereas that of FP(B) was unaffected. In contrast, the addition of 100 molar excess of a 25-bp non-specific oligonucleotide had no effect on nuclear factor binding to either FP(A) or FP(B) (Fig. 1, lane 7), whereas the addition of the unlabeled 249-bp CaRE fragment, inhibited protection of both regions (Fig. 1, lane 8). To test whether the protected region of FP(A) is due to the interactions of AP-1-transacting factors to this sequence, we performed DNase I protection analysis on the 249-bp fragment in the presence of purified c-Jun protein. As shown in Fig. 2, c-Jun binds to FP(A), although the boundary of the protected region is slightly different from that defined by the keratinocyte nuclear extract. Note that c-Jun did not interact with FP(B) nor with other potential cis-elements encoded by this fragment. The change in the footprinting pattern observed in the FP(B) region with the addition of c-Jun may be attributable to nonspecific effects caused by the relatively high levels of c-Jun protein in this assay.

In mobility shift experiments on the CaRE-encoding fragment, three retarded complexes were consistently observed (Fig. 3A). Complex II disappeared upon the addition of 60-fold molar excess of an oligonucleotide containing the AP-1 consensus sequence, suggesting that this complex was formed by interaction of AP-1 factors to FP(A). Complexes I and III disappeared when an oligonucleotide encoding the thyroid hormone response element was used as the competitor, suggesting that proteins related to members of the thyroid receptor superfamily interact with cis-elements located within the FP(B) region. These results were confirmed by repeating the gel shift assay on end-labeled fragments where the FP(A) and FP(B) regions were deleted individually from the original 249-bp CaRE-encoding fragment (Fig. 3B). One complex (Complex II) was formed with a fragment lacking the FP(B) region (CaREΔB) and was specifically competed by the AP-1 oligonucleotide but not by oligonucleotides encoding the thyroid response element, retinoic acid response element, or vitamin D3 response element. Two complexes (Complexes I and III) were observed with a CaRE deletion mutant lacking the FP(A) region (CaREΔA), and both complexes were removed upon the addition of oligonucleotides encoding hormone response elements. The stability of these complexes were unaffected by the addition of the AP-1 oligonucleotide. Taken together, these data suggest that members of the AP-1 family of transacting factors interact with FP(A) and that members of the steroid hormone superfamily can potentially interact with the FP(B) site.

**Calcium Induction Is Mediated Through the FP(A) Site**—To assess whether the sequences protected in FP(A) and FP(B) were functionally involved in mediating the calcium response, we employed a reporter construct consisting of the CaRE linked to a SV40 minimal promoter CAT construct. The CaRE.CAT construct has been shown to be activated by increasing concen-
trations of calcium in the medium (32). To test whether endo-
genous AP-1 factors were able to induce CAT activity from the CaRE.CAT construct, we added TPA to transfected keratino-
cytes. TPA is a potent inducer of AP-1 factors in keratinocytes and is able to activate CaRE.CAT in a dose-dependent manner (Fig. 4). Thus, both calcium and TPA are able to induce CAT activity from the CaRE.CAT construct.

To determine the contribution of each protected region to CaRE activity, we linked the fragments lacking either FP(A) or FP(B) to pA10CATz and tested their ability to induce reporter gene activity in the presence of calcium. Deletion of FP(B) did not affect calcium responsiveness while deletion of FP(A) completely abolished the calcium response (Fig. 5). This observation suggests that all of the calcium response mediated by the CaRE can be attributed to factors interacting with the sequences protected in FP(A).

Vitamin D₃ and Retinoic Acid Modulate Calcium Induction of the HK1 CaRE—To determine the function of the FP(B) region, various steroid hormones, including thyroid hormone, retinoic acid, vitamin D₃, and estrogen, were used to induce the activity of the 249-bp element in low calcium medium. These hormones by themselves were unable to induce CAT activity in a CaRE.CAT fusion construct (data not shown). To determine whether these hormones were able to modulate calcium induction of the CaRE, transfected keratinocytes were treated with each hormone in the presence of high calcium medium (0.35 mm). Thyroid hormone, retinoic acid, or estrogen had no effect on calcium induction (data not shown), but interestingly, vitamin D₃ was found to suppress calcium induction (Fig. 6). Since it has been observed that steroid hormone receptors are able to form heterodimers with other members of this superfamily (45), we asked whether the simultaneous addition of two hor-
mones could influence CaRE activity in the presence of activating levels of calcium. To assess this, thyroid hormone, retinoic acid or estrogen were added along with vitamin D₃ and calcium after transfection of the CaRE.CAT reporter construct. In combination, retinoic acid was able to reverse the suppression of calcium induction by vitamin D₃ (Fig. 7). Thyroid hormone and estrogen had no effect on vitamin D₃ suppression of the CaRE..

In order to show that hormonal modulation of CAT activity was specific to the CaRE, we tested the effects of vitamin D₃ alone or in conjunction with retinoic acid on the activity of another TPA-responsive promoter (pHIVLTR.CAT) in trans-
fected keratinocytes. The HIVLTR promoter is also inducible by calcium (data not shown), but its activity was only marginally suppressed by vitamin D₃, in contrast to the CaRE of HK1 (Fig. 7). Moreover, the coaddition of retinoic acid had no effect on the

**Fig. 4. Activation of the HK1 CaRE by TPA.** Primary mouse keratinocytes were transfected with the CaRE.CAT construct and after 48 h switched to the indicated medium. Lane 1, 0.05 mm calcium; lane 2, 10 ng/ml TPA; lane 3, 100 ng/ml TPA; lane 4, 1000 ng/ml TPA.

**Fig. 5. Comparison of the calcium response of the HK1 CaRE with that of the CaRE deletion mutants.** Primary mouse keratinocytes were transfected with CaRE.CAT, CaREΔA.CAT, and CaREΔB.CAT constructs and after 48 h switched to medium containing either 0.05 or 0.35 mm calcium (Ca²⁺).

**Fig. 6. Suppression of calcium induction of the HK1 CaRE by vitamin D₃ is dose-dependent.** Primary mouse keratinocytes were transfected with CaRE.CAT and after 48 h switched to medium containing 0.35 mm calcium and the concentrations of vitamin D₃ (1,25-(OH)₂D₃) indicated.

**DISCUSSION**

We have identified two adjacent footprinting regions within a 249-bp fragment that has recently been shown to confer calcium responsiveness to both the HK1 and SV40 promoters (32). No other protein binding regions were observed within this fragment. The 5’ most protected region, FP(A), encodes an AP-1-binding site and is the cis-element through which calcium activation is mediated. The adjacent protected region, FP(B), contains a hormone response element. While this site is not necessary for calcium activation, it does play a role in the modulation of the calcium response. We have shown that vita-
the coaddition of retinoic acid can antagonize the effect of vitamin D3. Independent transfection experiments. The HK1 promoter and immediate upstream sequences were unable to activate transcription of an HKLCAT reporter fusion construct in the absence of the 3′-CaRE (32). Interestingly, the HK1 promoter and immediate upstream sequences both proximal and distal to the promoter and that the 3′-CaRE element is functionally important for transcription of these genes. In this respect the AP-1 site identified in the HK1 promoter appears to be unique. Calcium-inducible enhancers have been described for a number of other genes (55–57) including Jun and Fos (58–60). To date, no common pathway has emerged, but there are several possible elements through which calcium can influence gene transcription. Transcription of c-fos for instance, can be induced by calcium through at least three different cis-elements (58, 59) but it remains to be seen which of these are invoked in keratinocyte differentiation.

Numerous studies have examined the expression of AP-1 factors in the epidermis. Transcripts for junB have been localized within the suprabasal, most-differentiated layers of the skin while c-jun mRNA appears to be localized to a subpopulation of basal cells (61). Fos protein can be detected in a few proliferating basal cells but is predominant in the cells of the upper granular layer (62). Moreover, a transgenic study employing a Fos-lacZ fusion gene, detected low levels of fos promoter activity in the basal layer rising to much higher levels in the spinous and granular layers (63). This observation suggests the tantalizing possibility of a gradient of fos expression that is invoked in keratinocyte differentiation.

The proximal footprint FP(A) encompasses sequences between +7895 and +7921 with respect to the start of HK1 transcription and is responsible for mediating the calcium activation of both homologous and heterologous promoters. This region encodes an AP-1 site, and we have inferred from our footprint and gel shift assays that AP-1 factors bind to this region to confer the calcium response. A second potential AP-1 site within the 249-bp fragment was identified by sequence homology comparison in the earlier study by Huff et al. (31) but appears to be non-functional in keratinocytes. This site was not protected in footprinting assays by proteins from keratinocyte nuclear extracts nor by purified c-Jun. Moreover, the CaREΔA construct which lacks the first AP-1 site within FP(A), but retains the second potential AP-1 site, was unable to induce CAT expression. The two AP-1 sites differ only in two positions, one of which occurs at a position relatively insensitive to substitutions (47). These results imply that AP-1 factors in keratinocytes can discriminate between these two sites, and it is possible that sequences neighboring the AP-1 site within FP(A) also contribute to nuclear factor binding to this region (48).

AP-1 sites have been identified in keratins K8 and K18 (49, 50) as well as in the promoter regions of the keratinocyte-specific, human papilloma viruses types 16 and 18 (51–54). An AP-1 site in the first intron of human K18 has been shown to be activated by coexpression of Fos and Jun (50). Similarly, Fos and Jun have been shown to bind to the AP-1 sites within the long control region of human papilloma viruses (51–54) where it is suggested that AP-1 sites are necessary but not sufficient for transcription. However, none of the above sites have been reported to be involved in calcium induction of these genes. In the case of HK1 transcriptional activation by the CaRE, one could envisage that perhaps Jun-Jun homodimers occupy the AP-1 element in FP(A) under low calcium conditions (i.e. a basal cell) to keep this gene switch off. As the calcium concentration increases in the differentiating keratinocyte, more Fos is induced, leading to a predominance of Fos-Jun heterodimers at the FP(A) site and a concomitant increase in HK1 gene transcription. Indeed, in preliminary studies, constitutive coexpression of c-fos was shown to activate CaRE.CAT in transfected keratinocytes in the absence of calcium, while cotransfection of c-jun suppressed calcium induction through this element.3

The distal footprint FP(B) defines a protected region between +7924 and +7948 with respect to the start of transcription. We have shown that although the FP(B) region is superfluous for calcium activation it is involved in the regulation of the FP(A) site. While these studies cannot reveal the exact nature of the interaction between AP-1 factors and steroid hormone receptors binding to the CaRE, a number of schemes have been

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3 B. Lu, unpublished results.
proposed that involve both protein-protein and protein-DNA interactions to account for the cross-talk between nuclear factor families (64). Both types of interactions would seem to be involved in the suppression of calcium induction by vitamin D3. At least some of this suppression appears to be mediated by receptor binding to the FP(B) site and subsequently interacting with AP-1 factors occupying the FP(A) site to modulate their activity. Since suppression by vitamin D3 could be observed in the CaREAB construct other pathways that do not involve the FP(B) site must be involved. These include direct effects of vitamin D3 on the promoters of AP-1-encoding genes or interactions with these components prior to their binding to regulatory elements. Indeed, it has been proposed that hormone receptors may associate with c-Jun or c-Fos in the absence of DNA (64), and, recently, Kerppola et al. (65) have shown that the glucocorticoid receptor can directly interact with both Fos and Jun through its zinc finger DNA-binding domain, effectively preventing them from binding to AP-1 sites and participating directly in transcriptional regulation. Equally interesting was our finding that retinoic acid was able to reverse the effects of vitamin D3 on the CaRE. It is not clear how this antagonism is mediated, but one possibility is that the activated retinoic acid receptor may compete with the vitamin D3 receptor for their common coactivator, RXR (66-69). In any case, further studies are necessary to determine whether this antagonism by retinoic acid is physiologically relevant in vivo.

The expression of HK1 mRNA is tightly regulated and restricted to the spinous layer of the epidermis which can be considered as an intermediate state between the loss of proliferative potential and cell death. Since calcium can inhibit proliferation of keratinocytes and promote their differentiation and vitamin D3 can induce cornification of cells already undergoing differentiation, we propose that calcium promotes HK1 gene transcription through the up-regulation of AP-1 factors and their subsequent binding to the FP(A) site, concomitant with or soon after the cell enters the differentiation pathway. HK1 transcription would then be maximal in the mature spinous layer cell where retinoic acid levels are high enough to antagonize the action of vitamin D3 keeping the FP(A) site responsive to calcium activation. Later, as the keratinocyte differentiates into a granular layer cell, where vitamin D3 levels are maximal and retinoic acid levels are at their lowest, the activity of the FP(A) site would be suppressed and HK1 transcription down-regulated. Thus, the interaction of these modulators with the CaRE can account for the restricted expression of HK1 observed in vivo.

In summary, we have demonstrated the interaction of AP-1 factors with FP(A) and that of vitamin D3 and retinoic acid through their receptors with FP(B). The complex interplay between each of these factors serves to restrict expression of HK1 to the spinous cell during epidermal differentiation. We conclude therefore that the CaRE of HK1 functions as a differentiation stage-specific enhancer.
Regulation of Human Keratin 1