Identification of Control Elements 3′ to the Human Keratin 1 Gene That Regulate Cell Type and Differentiation-specific Expression*

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Carol Ann Huff, Stuart H. Yuspa§, and Dean Rosenthal§

From the Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

To define DNA regulatory elements that mediate the response of the keratin 1 (K1) gene to Ca2+-induced differentiation, regions spanning the 5′- and 3′-flanking sequences, coding regions, and introns from the human K1 gene were cloned into vectors containing the chloramphenicol acetyltransferase (CAT) reporter gene and transfected into cultured mouse keratinocytes. A 4.3-kilobase (kb) region located 3′ to the K1 gene stimulated CAT activity in response to increasing Ca2+ concentrations from 0.05 mM (basal cells) to 1.2 mM (differentiated cells). The 4.3-kb fragment was also active in human epidermal cells but inactive in NIH 3T3 cells and primary mouse fibroblasts. Deletion analysis localized the activity to the terminal 1682 base pairs (bp) of the flanking sequence which retained Ca2+-sensitivity in epidermal cells but was not active in mesenchymal cells. Removal of a 207-base pair element created an enhancer which was active in both epidermal and mesenchymal cells but was still Ca2+-inducible. Further deletions identified two elements which functioned synergistically to give maximal Ca2+-sensitive activity. Stably transfected epidermal cell lines expressed CAT under the direction of these elements when grafted onto nude mice to reconstitute an intact epidermis. Previously reported keratin regulatory motifs were not contained in the 1682-bp fragment, but an AP-1 site was identified in one of the synergistic subunits.

Epidermal differentiation is a complex process that encompasses the expression and organization of unique cytoskeletal proteins and the cessation of proliferation. Although a number of the molecular changes associated with this process have been characterized, the factor or factors which regulate the process remain unknown. One of the earliest and most clearly inducible alterations in differentiating keratinocytes is a change in the pattern of keratin gene expression. Proliferating keratinocytes in the basal layer express keratins K5 and K14, but these keratins are repressed in differentiating keratinocytes and a new pair of keratins, K1 and K10, are induced (1-3).

Keratins are regulated primarily at the level of transcription, although posttranscriptional modification has been identified (1, 2, 4-7). In cultured keratinocytes the expression of mRNA and protein for keratins 1 and 10 can be induced by specific concentrations of extracellular Ca2+ (8). The induction of keratin 1 expression requires protein synthesis, as treatment with cycloheximide inhibits keratin 1 transcription in the appropriate Ca2+ medium (8). This suggests that an inducible protein is involved in the regulation by Ca2+. Many eukaryotic genes exhibiting tissue-, developmental-, or differentiation-specific patterns of expression have been shown to be regulated by cis-acting elements. These elements often function as binding sites for trans-acting factors which either increase or decrease the expression of their associated genes. Such elements have been identified either 5′ (9-14), within introns (15), or 3′ to keratin genes (16). In light of the epidermal and differentiation-specific pattern of keratin 1 expression, it seemed likely that similar elements would be involved in its regulation.

In previous studies, a 10.8-kb fragment encoding human keratin 1 (HK1) had been isolated from a λ genomic library (17). This fragment included the entire coding region as well as 1200 bp of the 5′- and 4300 bp of 3′-flanking sequences (17). When this genomic clone was introduced into transgenic mice, HK1 was expressed only in the epidermis (3). Expression of HK1 was first detected in the developing epidermis of 15-day embryos, suggesting that the clone contained sequences which determined developmental specificity (3). Within the epidermis of these transgenic mice, endogenous mouse keratin 1 was confined to the suprabasal cell compartment, whereas HK1 was expressed in most suprabasal cells, but also was detected in some basal cells. This suggested that the transgene was not as tightly regulated as the endogenous mouse keratin 1. When primary keratinocyte cultures were prepared from these transgenic mice, MK1 and HK1 could be induced by culturing in Ca2+ > 0.10 mM (18). Although both genes were Ca2+-inducible, they had different optima, with mouse K1 being maximally induced in 0.12 mM Ca2+ and human K1 in 0.6 mM Ca2+. These studies indicated that the 10.8-kb genomic fragment included sequences which targeted HK1 expression primarily to the suprabasal epidermis and which responded to Ca2+-induced differentiation in vitro.

We now describe the localization of a tissue-specific and Ca2+-inducible DNA regulatory region within this HK1 genomic fragment. Transient transfection of primary mouse and human keratinocytes with fusion constructs of different regions of HK1 and the chloramphenicol acetyltransferase (CAT) gene has shown that this regulatory segment is located within a 4.3-kb fragment located 3′ to the HK1 gene. Deletion studies reveal an array of regulatory elements in the terminal

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‡ To whom requests for reprints should be addressed. Tel.: 301-496-2162; Fax: 301-496-8709.

§ Present address: Dept. of Biochemistry and Molecular Biology, Georgetown University School of Medicine, Washington, D. C. 20007.

The abbreviations used are: kb, kilobase(s); HK1, human keratin 1; MK1, mouse keratin 1; SV40, simian virus 40; bp, base pairs; CAT, chloramphenicol acetyltransferase; SVE, simian virus 40 enhancer; KGM, keratinocyte growth medium; EMEM, eagle’s minimal essential medium; EGF, epidermal growth factor.
1682 bp of this 3'-flanking sequence. There appear to be two enhancers which function synergistically, one of which confers Ca\textsuperscript{2+} inducibility, and a negative element that is important in cell type specificity as its removal leads to activity in both epidermal and mesenchymal cells.

**MATERIALS AND METHODS**

**Plasmid Constructions**—Molecular cloning followed established protocols as described (19). pAlOCAT, containing the SV40 early promoter, Thr 9 chloramphenicol acetyltransferase (CAT) gene, and small l antigen splice site and polyadenylation signal was used as the original for all cloning vectors (20). pGem7ZCAT(+) and pGem7ZCAT(+) and pGemZ7CAT(+) and pGemZ7CAT(+) were derived from a human \( \lambda \) Charon 4A library (17) (Fig. 1). The 5' HK1-CAT fusion constructs were generated by cloning either the 5' EcoRI-PstI HK1 fragment (containing 1 kb of the 5'-flanking sequence) or the 5' EcoRI-KapI HK1 fragment (containing 1.2 kb of the 5'-flanking sequence), adjacent to the 1.8 kb BglII-BamHI CAT fragment in pGem3CAT(+) (Fig. 1, A and B). Both HK1 5' sequences were cloned 5' to CAT in the sense orientation.

The coding region HK1-CAT fusion construct was generated by cloning the 6.4 kb EcoRI-BamHI HK1 fragment into the BamHI site of pGemZ7CAT, placing HK1 3' to the 1.8 kb CAT fragment in the sense orientation (Fig. 1C).

The 3' HK1-CAT fusion construct (p4.3CAT) was generated by cloning the HK1 10.8 kb BglII-EcoRI fragment into pGem3, digesting with BamHI to remove the 5' and coding sequences, and ligating to the 1.8 kb BglII-BamHI CAT fragment in pGem3CAT(+) (Fig. 1, A and B). Both HK1 5' sequences were cloned 5' to CAT in the sense orientation.

By using known restriction sites within p4.3(+), a series of deletions was made. Partial digestion of p4.3(+), followed by religation, generated p1700. Complete digestion with the same enzymes and ligation created p1580. Digestion with BglII alone followed by religation generated p580. To complete the series of overlapping constructs, the internal 1.0 kb BglII fragment was subcloned into the pGem7-CAT(+) vector.

Digestion of p1700 with XbaI and SmaI generated a fragment containing the CAT cassette and 1475 base pairs of the human K1 terminations was made. Partial digestion of p4.3(+) with BglII and BamHI, HincII and NsiI sites to create p1475. Partial digestion with NsiI created p580, followed by religation created p1700. To complete the series of over-

Control Elements—Primary mouse fibroblasts were prepared as described (21, 25). Cultures were maintained for 7 days in 1.2 mM Ca\textsuperscript{2+}, EMEM medium prior to transfection. Cells were trypsinized and plated at a density of 0.5 \( \times \) 10\textsuperscript{6}/60-mm dish 14 h prior to transfection. Cells were refed 4 h prior to transfection. DNA was introduced by Ca\textsuperscript{2+} phosphate co-precipitation for 4 h followed by a 2-min 25\% dimethyl sulfoxide shock and refueling with 1.2 mM Ca\textsuperscript{2+}, EMEM. In some experiments, the human fibroblast cell line YDF (26) and NIH 3T3 cells were used as recipients for DNA transfection using the same protocols and conditions described for primary mouse fibroblasts.

**Normal Human Epidermal Keratinocytes**—Cultures of epidermis from breast skin were obtained from Clonetics (San Diego, CA) and cultured in serum-free keratinocyte growth medium (KGM) (0.05 mM Ca\textsuperscript{2+}) until near confluence. Cells were trypsinized and plated at 5 \( \times \) 10\textsuperscript{6} cells/60-mm dish. Fourteen hours after plating, cells were switched to EMEM containing 0.05 mM Ca\textsuperscript{2+}, 10 mM K\textsuperscript{+}, and 8% fetal calf serum. After 4 h, cultures were transfected using the same protocol as for mouse keratinocytes.

Sixteen hours after transfection, cells were switched back to KGM (0.05 mM Ca\textsuperscript{2+}) for 24 h prior to challenge in KGM with higher Ca\textsuperscript{2+} medium.

**CAT Assays**—Cultures were washed three times with phosphate-buffered saline (without Ca\textsuperscript{2+} or Mg\textsuperscript{2+}) and frozen on dry ice. Cells were freeze-thawed three times in 0.1 M Tris, pH 7.7, to disrupt the plasma membranes and release the soluble proteins. The soluble fraction was isolated and protein concentration measured using the Bradford Reagent (27) (Bio-Rad); absorbance at 595 nm for samples, as well as for bovine serum albumin standards, were determined in duplicate on a microtiter plate reader. Individual culture extracts were then normalized so that equal amounts of protein were analyzed from each dish. Enzymatic CAT activity was measured using the two-phase fluor diffusion assay as described (28) with the following modifications. 1) [\( \text{H} \)]Acetyl-CoA (Du Pont-New England Nuclear) was used at a 1:35 (v/v) dilution with unlabeled acetyl-CoA (Pharmacia LKB Biotechnology Inc.) to increase the sensitivity, and 2) 10 h was chosen as the reference point for all analyses, since the reaction rate was within the linear range at this time point. Nevertheless, in each experiment a complete time course of activity was performed. All samples were counted on a LKB Scintillation Counter. In all experiments CAT assays were analyzed relative to the values for pSV2CAT transfectants in the same experiments. All experiments were performed at least three times and in duplicate for each construct.

Sequence of p1700 and Comparison to Known Regulatory Elements—The nucleotide sequence of p1700 was obtained using dinucleotide sequence analysis methodology (Sequenase, U.S. Biochemicals). Both DNA strands of p1700 were sequenced yielding 1828 nucleotides. Comparison to published sequences in GenBank was performed using the University of Wisconsin Genetics Control Group Software Package.
RESULTS

Enhancer Activity of the 10.8-kb Human Keratin 1 Fragment in Mouse Keratinocytes—In light of the fact that the 10.8-kb human K1 genomic fragment contained sufficient sequence information to respond to Ca\(^{2+}\) in primary transgenic keratinocytes (18), CAT fusion constructs derived from different regions of the human K1 gene were then tested in transient transfections (Fig. 1). These constructs were derived from two overlapping 1.1- and 1.2-kb 5' regions (EcoRI-PstI and EcoRI-KpnI), one 6.4-kb region containing most of the coding sequence and all the introns (EcoRI-BamHI), and one 4.3-kb 3' non-coding region (BamHI-EcoRI). These constructs spanned the entire coding sequence, all introns, 1.2 kb of immediate 5'-flanking sequence, and 4.3 kb of immediate 3'-flanking sequence (Fig. 1). None of the HK1 5'-flanking region or -coding region constructs showed any significant enhancer activity above background, when compared with either Tris-Cl or the enhancerless constructs. In contrast, the 4.3-kb 3'-non-coding region, as well as the positive control pSVECAT, showed strong activity. However, although pSVECAT displayed significant enhancer activity in all Ca\(^{2+}\) concentrations tested, the HK1 3' region was primarily active in higher Ca\(^{2+}\) concentrations (Table I). For example, the absolute activity of p4.3(+) was significantly lower (30%) than that of pSVECAT in 0.05 mM Ca\(^{2+}\) (p = 0.007, n = 5; two-tailed Fisher's exact test). Following a Ca\(^{2+}\) shift from 0.05 to 1.2 mM, pSVECAT displays a 2.9-fold induction in activity, whereas p4.3(+) shows nearly a 15-fold increase in activity. The absolute activity of p4.3(+) is 1.5-fold higher than pSVECAT at this concentration of Ca\(^{2+}\). The same 4.3-kb 3' region of HK1 cloned in the reverse orientation 5' to CAT, p4.3(-), also shows a substantial Ca\(^{2+}\)-dependent induction, although the absolute level of activity was lower than that of p4.3(+).

Fig. 2 shows the Ca\(^{2+}\)-sensitive activity of pSVECAT compared to p4.3(+). Both p4.3(+) and pSVECAT increase in activity with increasing Ca\(^{2+}\) concentrations in all experiments, although p4.3(+) always increases to a greater relative and absolute extent. Incrementally, the largest change in expression occurs between 0.12 and 0.6 mM Ca\(^{2+}\), although activity continues to increase between 0.6 and 1.2 mM Ca\(^{2+}\).

The low level of activity of p4.3(+) in 0.05 mM Ca\(^{2+}\) medium is not unexpected, since K1 has been shown to be expressed at low levels in 0.05 mM Ca\(^{2+}\) medium (8), and the HK1 transgene is detected in some basal cells in 0.05 mM Ca\(^{2+}\) cultures of transgenic epidermis (18).

Since the 10.8-kb HK1 fragment is exclusively expressed in the epidermis of transgenic mice (3), we determined whether the 4.3-kb Ca\(^{2+}\)-responsive element is cell type-specific. NIH 3T3 cells were transfected with either pSVECAT, p4.3(+), or pA10CAT. The SV40 enhancer displays significant activity in 3T3 cells (not shown) as it does in a wide variety of cells that have been tested. In contrast, the HK1 enhancer shows no detectable activity above background in NIH 3T3 cells, even though these cells were cultured in medium containing 1.8 mM Ca\(^{2+}\). The SV40 enhancer, but not the HK1 enhancer, was also active in primary mouse fibroblasts in 1.2 mM Ca\(^{2+}\) medium. In contrast, both pSVECAT and p4.3(+) were active in human keratinocytes, and CAT activity directed by p4.3(+) sequences increased 1.6-fold when cells were switched from 0.05 to 0.6 mM Ca\(^{2+}\) (not shown, see below).

Deletion Analysis—To localize the region or regions of enhancer activity within the 4.3-kb sequence, a series of deletions were made in p4.3(+) using known restriction sites. Transfection analysis of the original construct (p4.3(+)), and the initial deletions are shown in Fig. 3 and represent CAT assay data from cells in 0.05 and 0.6 mM Ca\(^{2+}\) medium for 48 h. Analysis of these constructs indicates that the Ca\(^{2+}\)-dependent enhancer activity of the 4.3-kb fragment is located in the distal 1682 bp (determined by sequence), as seen in construct p1700 (determined by mobility). p1700 retains both the Ca\(^{2+}\) inducibility (4-5-fold) and the absolute level of activity (1.4-fold greater than SVE CAT) seen in the parent construct. Each of the other deletions has lost most or all of the enhancer activity and Ca\(^{2+}\) inducibility. The sequence for construct p1700 is shown in Fig. 4 and will be discussed below.

![Diagram of recombinant plasmids with fragments of the human keratin 1 (K1) gene.](image)

**FIG. 1. Construction of recombinant plasmids with fragments of the human keratin 1 (K1) gene.** The 10.8-kb genomic clone is depicted at the top of the diagram. Each of the fragments shown in A-D was fused to CAT in pGem3 (A, B, and D) or pGem7Z (C). The two 5'-flanking fragments (A and B) were cloned 5' to CAT in the correct orientation, whereas the coding region and 3'-flanking region fragments (C and D) were cloned 3' to CAT in the correct orientation (p4.3(+)). The 3'-flanking region (D) was also cloned 5' to CAT in the opposite orientation (p4.3(-)).

**TABLE I**

| Construct | Relative CAT activity in primary mouse keratinocytes* |
|-----------|------------------------------------------------------|
|          | n Induction, 1.2 0.05 mM Ca\(^{2+}\)                   |
| n 0.05 | |
| pSVECAT   | 4 2.4 5 2.9                                         |
| p4.3 (+)  | 4 6.8 10 14.5                                       |
| p4.3 (-)  | 3 5.1 ND                                             |

* n, number of replicate experiments; ND, not determined.

**FIG. 2. Ca\(^{2+}\) dose-response of p4.3(+) and pSVECAT.** Primary mouse keratinocytes were transfected in 0.05 mM Ca\(^{2+}\) medium with either pSVECAT (hatched boxes) or p4.3(+) (solid boxes), then switched to the indicated concentrations of Ca\(^{2+}\) for 48 h. CAT activity was assayed as described under "Materials and Methods."
that, like p1700, the Ca\(^{2+}\) inducibility of p1475 is specific for mouse keratinocytes. This construct is completely inactive in the nonepidermal keratinocytes. Fig. 5 also shows that whereas p1700 is active in mouse epidermal cells as a monolayer in 0.60 mM Ca\(^{2+}\) medium, activity is expressed as a percentage, with the activity of pSVECAT in 0.60 mM Ca\(^{2+}\) medium being 100%.

To determine whether p1700 retains the cell type specificity of p4.3(+), p1700 was transfected into cells of nonepidermal origin, including primary mouse fibroblasts, NIH 3T3 cells, and a human fibroblast cell line YDF. In each case, the activity of p1700 was normalized against the activity of pSVECAT. Since each of these cell types has a Ca\(^{2+}\) optimum of >1 mM for growth, fibroblast transfectants were compared with mouse keratinocytes in medium containing 1.2 mM Ca\(^{2+}\). Fig. 5 shows that whereas p1700 is active in mouse epidermal keratinocytes, this construct is completely inactive in the three mesenchymal cell types examined. Furthermore, p1700, but not pSVECAT, was inactive when mesenchymal cells were cultured in medium containing concentrations of Ca\(^{2+}\) ranging from 0.05 to 1.4 mM (data not shown). Thus, p1700 is Ca\(^{2+}\)-inducible only in keratinocytes. Fig. 5 also shows that p1700, like p4.3(+), is active in human as well as mouse keratinocytes. The apparent lower activity in human versus mouse keratinocytes is due to a lower ratio of p1700/pSVECAT directed activity, rather than lower absolute activity.

When the 3' region of p1700 is deleted, the resulting construct, p1475, showed increased activity in mouse keratinocytes compared with both p1700 and pSVECAT (Fig. 6), suggesting that the terminal 207-bp region contains a strong negative regulatory element. Fig. 6 also shows that although p1475 has significant activity in 0.05 mM Ca\(^{2+}\) (comparable with that of pSVECAT in 0.6 mM Ca\(^{2+}\)), the activity of p1475 still increases 4-5-fold when cells are switched to 0.6 mM Ca\(^{2+}\). Since p1475 exhibits significant activity under conditions in which endogenous K1 is not expressed (0.05 mM Ca\(^{2+}\)), we were interested in determining the activity of this construct in nonepidermal cells. Fig. 7 shows that p1475, but not p1700, is active in NIH 3T3 cells. Similar results were obtained for primary mouse fibroblasts and YDF cells (not shown). In NIH 3T3 cells, p1475 exhibited activity comparable with that of pSVECAT in medium containing concentrations of Ca\(^{2+}\) ranging from 0.05 to 1.40 mM, suggesting that, like p1700, the Ca\(^{2+}\) inducibility of p1475 is specific for the keratinocytes.

Removal of the internal Neil-NsiI fragment from p1475 yielded a construct, p580, with activity comparable with p1475 in both epidermal and nonepidermal cells, whereas the reciprocal construct containing only the deleted 895-bp fragment had minimal activity in mouse keratinocytes in all concentrations of Ca\(^{2+}\) (Fig. 8). When the 580-bp recombinant segment was analyzed as its constituent 422- and 158-bp elements, neither was capable of enhancing CAT to the same level as p580, and the segments functioned synergistically rather than additively (Fig. 8). The 422-bp element is responsible for the Ca\(^{2+}\) inducibility of the enhancer array, since p422 is induced 8-10-fold in 0.6 mM Ca\(^{2+}\), whereas p158 maintains essentially constant activity under all conditions.

Since at least two elements within p1700 are necessary for maximal enhancer activity in mouse keratinocytes, additional 5', 3', and internal deletions in p580 were constructed in an attempt to further localize the enhancer activity (Fig. 9). Neither p514 nor p429 retains the activity of p580. Removal of as few as 66 bp from the 3' end (p514), or 151 bp internally (p429), resulted in a significant reduction in activity, although Ca\(^{2+}\) inducibility was maintained. A 120-bp 5' deletion (p460) from p580 resulted in the loss of basal activity as well as Ca\(^{2+}\) inducibility, demonstrating the importance of this region in the functioning of the enhancer elements. Preliminary findings using DNA footprinting techniques and nuclear extracts from keratinocytes have shown a 30-bp region of strong protection within the 120-bp deleted segment, further supporting the importance of this region in the Ca\(^{2+}\)-inducible enhancer activity (not shown).

As p580 contains the smallest region with maximal activity, it was employed to assess the functionality of the element in all orientations and positions. The 580-bp region was cloned 5' and 3' to the CAT gene in both the sense and antisense orientations. These four constructs were transfected into primary mouse keratinocytes and CAT activity measured after culturing cells in 0.05 or 0.6 mM Ca\(^{2+}\) medium. Fig. 10 shows that the basal activity directed by the 580-bp element is greater in the 5' orientation and relative induction by Ca\(^{2+}\) is slightly better in the 5' sense orientation. However, 3' constructs remain active and Ca\(^{2+}\)-inducible in both sense and antisense orientations. These experiments show that this element functions in all four orientations and positions and thus fits the classification as an enhancer.

Stably transfected keratinocyte cell lines were created by co-transfection of the cell line BK-1 with either the enhancerless pGem7ZCAT or p4.3 or p580 together with pSV2NEO. Cell lines were selected from each of the plasmids and first analyzed for CAT activity in vitro. These cell lines were then grafted onto nude mice. Twenty-eight days after grafting, the skin at the graft site was harvested, and whole tissue extract was prepared as described and analyzed for CAT activity. CAT activity was not detected in normal nude mouse skin (0 positive grafts/5 graft recipients) or BK-1 cells stably transfected with pGem7ZCAT (0/10 animals). However, constructs p4.3, (7/17 animals) and p580 (11/12 animals) yielded skin grafts with readily detectable CAT activity. This indicates that these elements are capable of functioning as enhancer elements in vivo in epidermis, although the epidermal specificity cannot be assessed by these studies.

**DISCUSSION**

We have demonstrated that a 4.3-kb fragment located immediately 3' to the human keratin 1 gene contains genetic elements that direct the synthesis of CAT from the SV40 early promoter in both mouse and human epidermal cells, but not in NIH 3T3 cells or primary mouse or human fibroblasts.
FIG. 4. Nucleotide sequence of the 1700-bp Ca**+-inducible epidermal-specific regulatory region 3' to human keratin 1. The 1700-bp (based on mobility) genomic fragment was sequenced as described under "Materials and Methods" and compared with GenBank sequences for regions of homology. Regions I and II (sparsely stippled) confer Ca** inducibility and maximal activity, respectively. Asterisks underly sequence 100% homologous to AP-1 binding site, filled circles denote sequence 100% homologous to a TGFP consensus negative response element, and arrows underscore imperfect direct repeats. Region III (densely stippled) denotes negative element. Open circles underly sequence 100% homologous to keratinocyte consensus sequence (38).

No other DNA regulatory elements were found within a 10.8-kb fragment containing the human keratin 1 gene. When transfected into mouse keratinocytes, the 4.3-kb BamHI-EcoRI human K1 fragment permits Ca**+-sensitive induction of CAT activity when situated either 3' to the gene (in the correct orientation) or 5' to the gene (in the inverse orientation), thus fitting the definition of an enhancer element. However, the 3' construct (p4.3(+)) was more active in mouse epidermal cells in 0.6 and 1.2 mM Ca**+ than both the 5' construct (p4.3(-)) and the SV40 enhancer construct (pSVECAT). Although elevated Ca**+ conditions enhance the expression of pSVECAT to a limited extent, the dose-response to Ca**+, the time course for induction (not shown), and the magnitude of the induction differed between p4.3(+) and pSVECAT. Since pSVECAT and p4.3 contain identical signals for RNA and protein processing, this differential expression most likely occurs at the level of transcription. Thus the HK1 enhancer appears to direct a specific response to Ca**+, as well as cell type specificity.

Although the initial localization of transcriptional enhancers was 5' to the genes they regulate, subsequent investigations have identified certain elements within introns and 3' to their associated genes, including genes encoding β-globin, γ-globin, α-interferon, T cell receptor α and β chains, immunoglobulin heavy chains, CD2, and others (29-35). Of particular interest to this study is the recent report of a 3' enhancer element in association with the gene encoding EndoA, a developmental-specific type I1 cytokeratin (16). Unlike the EndoA enhancer element which is comprised almost entirely of six repeated elements with homology to PEA3, there do not appear to be regions of homology to PEA3 in the 3' HK1 enhancer. Nevertheless, both elements are
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FIG. 5. Cell type specificity of p1700. p1700 was transfected into the five cell types indicated using calcium phosphate co-precipitation. All cells were cultured in 1.2 mM Ca²⁺ medium for 48 h and analyzed for CAT activity. For each cell type, the results are expressed as a percentage of pSVECAT activity (100%).

FIG. 6. Identification of a 207-bp negative regulatory element. Deletion of the 207-bp SnaBI-EcoRI fragment from p1700-created p1475. Primary mouse keratinocytes were transfected with 20 μg of the indicated constructs. Cells were cultured and analyzed for CAT activity following 48 h in 0.05 mM Ca²⁺ (slashed bars) and in 0.60 mM Ca²⁺ (open bars) medium. The activity of pSVECAT in 0.60 mM Ca²⁺ medium is 100%. Activity of the other constructs is expressed as a percentage of pSVECAT in 0.60 mM Ca²⁺ medium.

FIG. 7. Loss of cell type specificity with removal of the 207-bp SnaBI-EcoRI fragment. Twenty μg of the indicated plasmids were transfected into NIH 3T3 cells as described under "Materials and Methods." The activity of each construct is expressed as a percentage of pSVECAT activity in these cells.

FIG. 8. Identification of two positive elements within p1475. p1475 was analyzed by deletion analysis using the restriction sites indicated in the top diagram. This fragment was analyzed in three pieces and with an internal deletion, indicated by the dashed lines which denote deleted sequence. Twenty μg of each plasmid was transfected into separate cultures of primary keratinocytes as in all previous experiments. Cells were cultured in 0.05 mM Ca²⁺ medium or in 0.60 mM Ca²⁺ medium for 48 h and analyzed for CAT activity which is expressed as a percentage of pSVECAT activity in 0.60 mM Ca²⁺ medium.

FIG. 9. Further deletions of p580 reduce activity. Deletions were made in p580 using the restriction sites shown in the top diagram. The resulting constructs were named according to the length 3'-flanking sequence inserted into the CAT vector. Twenty μg of each construct was transfected into primary keratinocytes and analyzed for CAT activity following culture in 0.05 mM (slashed bars) Ca²⁺ medium or 0.60 mM (open bars) Ca²⁺ medium for 48 h. Results are expressed as a percentage of pSVECAT activity in 0.60 mM Ca²⁺ medium.

located 3' to type II keratins and appear to function as enhancers. At this time we cannot exclude the possibility that the enhancer we have characterized is a control region for a gene located further downstream. This would imply that the downstream coding sequence would be expressed in an epidermal-specific and Ca²⁺-inducible fashion. It is known that HK1 is clustered with other type II keratin genes on human chromosome 12 (36, 37).

Other studies have reported putative regulatory domains that may be involved in the expression of endogenous or viral genes in keratinocytes (10, 16, 38-45). These studies have identified regulatory sequences in conjunction with genes encoding human keratin 14 (5'-GCCTGAGGC-3'), a human K14 pseudogene, and XK81A1, an embryonic epidermal keratin gene in Xenopus laevis which is regulated by a factor called KTF-1 (12, 41, 46). A frequently occurring epidermal consensus sequence, 5'-AAPuCCAAA-3', has also been re-
FIG. 10. Position and orientation independence of p580. The 580-bp fragment was introduced into pGen7ZCAT in the sense and antisense orientations both 5′ and 3′ to CAT as indicated by the arrows in the left of the diagram. Each arrow is directed 5′ to 3′ with respect to the endogenous position of p580 in the 3′-flanking sequence of HK1. These plasmids (20 μg) were transfected into primary keratinocytes, cultured in 0.05 mM Ca2+ medium (slashed bars) or 0.60 mM Ca2+ medium (open bars) for 48 h, and analyzed for CAT activity. The results are expressed as a percentage of pSVE-CAT activity in 0.60 mM Ca2+ medium.

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To suppress heterologous enhancers or promoters (49, 50, 56, 59), To address the possibility that the 207-bp fragment might represent a silencer, it was cloned into pSVE-CAT. When it did not affect the level of SV40-directed CAT activity, it was considered to be involved in determining the absolute level of expression of HK1 is unclear, since neither HK1 nor MK1 is known to be involved in determining the absolute level of expression.

The significance of this finding is uncertain. In addition, p158 contains a region which also has 60% homology to nucleotides 66-75, 313-322, 1444-1453, and 1452-1461. A similar degree of homology was identified in assessing the KTF-1 consensus binding sequence (ACCCTGAGGCT), an imperfect palindrome which also has 60% homology to nucleotides 66-75 (14). Although the KTF-1 binding sequence and the keratinocyte HK14 palindromic sequence of p1475 reveals the presence of an AP-1 site as indicated by the arrows in the left of the diagram. The results are expressed as a percentage of pSVE-CAT activity in 0.60 mM Ca2+ medium.

To better define the elements responsible for calcium inducibility, additional deletions were made in p1475. These deletions identified two elements, p422 and p158. Neither element alone is sufficient to drive CAT expression to the same level as p1475. However, these elements appear to be interdependent and function synergistically as p580 exhibits the same pattern of expression as p1475. Such synergy has been identified previously for the 5′ human keratin 14 regulatory sequences, Drosophila homeobox elements, and skeletal α-actin (46, 63-65). Unlike the human keratin 14 elements, where neither element in isolation was able to drive expression (46), the human keratin 1 elements function weakly in isolation and are each able to enhance CAT expression to some degree.

Each of the HK1 enhancer elements appears to have a distinct function. The 422-bp proximal element seems to mediate induction by Ca2+, since both p422 and p580 are strongly induced when cells are switched from 0.05 to 0.6 mM Ca2+. The importance of this element was further illustrated and localized by deleting the 5′ most 120 bp of p580. This deletion yields a construct, p460, which is essentially inactive. Preliminary DNA footprint analysis has revealed a distinct 30-bp region of protection within the deleted 120-bp segment, strongly supporting the role of this region in trans-activation of the heterologous promoter. Analysis of the nucleotide sequence of p422 reveals the presence of an AP-1 site as indicated in Fig. 4. However, the contribution of this site to the regulation of K1 is unclear, since neither HK1 nor MK1 is induced by phorbol esters. The distal 158-bp element appears to be involved in determining the absolute level of expression since partial or complete deletion of this region from p580 leads to a reduction in activity but not loss of calcium inducibility. Further analysis of the nucleotide sequence of the p158 identified the presence of three repeats of an eight-nucleotide consensus sequence, GGNTGNGG (Fig. 4). The significance of this finding is uncertain. In addition, p158 contains a consensus sequence for the TGFβ inhibitory element which is conserved in a number of TGFβ-inhibited genes (66). It has been reported that TGFβ inhibits HK1 expression at high concentrations (67).

In an attempt to identify regions of homology to known
Ca\textsuperscript{2+}-responsive elements, the nucleotide sequence of the HK1 enhancer region was compared with known Ca\textsuperscript{2+}-responsive consensus sequences. Ca\textsuperscript{2+}-inducible enhancers have been described for the rat prolactin gene and the c-fos gene (68-70). In the latter case, the cyclic AMP-induced regulatory protein mediates this effect through its interaction with the cAMP response element (71). Within the proximal enhancer region (p22), there are two elements in tandem with six out of eight nucleotide sequence homology to the cAMP response element. However, cAMP is not induced by Ca\textsuperscript{2+} in mouse keratinocytes nor does cAMP induce differentiation (72). A similar comparison with the 1,25-(OH\textsubscript{2}) vitamin D\textsubscript{3} response element revealed no regions of significant homology within the HK1 enhancer elements (73). The lack of additional homology to calcium-responsive elements may not be surprising as the induction may be related to the Ca\textsuperscript{2+}-induced differentiation rather than to the Ca\textsuperscript{2+} itself.

Although transient transfections provide insight into the functional activity of DNA regulatory elements, they only give information on the in vitro activity of various sequences. That these 3'-regulatory elements were also active in vivo was suggested by the detection of CAT activity in skin grafts of neonatal hamsters with the HK2 construct. Since the enhancerless pGM7ZCAT construct was inactive in vivo, the studies show that the elements p4.3 and p5.80 are functionally active within the epidermis in vivo. Unfortunately we are unable to address the questions of specificity or differential expression with the current methods.

In summary, these experiments have identified three regulatory elements 3' to the human keratin 1 gene; two function synergistically in regulating Ca\textsuperscript{2+} inducibility, whereas the third appears to function in tissue specificity. Additional studies are needed to fully characterize these elements and the trans-acting factors which interact with them. These elements function both in vitro and in vivo and begin to address the question of cis-acting elements which may be involved in gene expression during epidermal differentiation.

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