Review paper

KERATINOCYTE DIFFERENTIATION AND ACTIVATION

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SUMMARY

To understand properly the various phenotypes associated with hereditary disorders due to abnormal keratinization, we need to understand the physiology of keratinocytes and the role of the proteins that keratinocytes synthesize to perform their function. There are two physiologic pathways open to keratinocytes, differentiation and activation, each with characteristic function and battery of proteins produced. The biologically active substances can be for didactic purposes categorized into four groups: signaling molecules, receptors, transducing molecules and transcription factors.

KEY WORDS

keratinocyte, differentiation, activation, signaling molecules, receptors, transducing molecules, transcription factors

To understand properly the various phenotypes associated with hereditary disorders due to abnormal keratinization, we need to understand the physiology of keratinocytes and the roles of the proteins that keratinocytes synthesize to perform their function. There are two physiologic pathways open to keratinocytes, differentiation and activation, each with characteristic function and battery of proteins produced.

Keratinocyte differentiation is associated with changes in gene expression, specifically of keratin genes, keratins being the most abundant epidermal proteins. The epidermis is composed of ten to twenty layers of keratinocytes, the predominant cell type of this tissue. During normal epidermal differentiation, four types of keratinocytes can be distinguished through their phenotypic and biochemical properties: basal, spinous, granular, and cornified [1][2]. Basal cells are characterized by their contact with the basement membrane, mitotic activity, and the expression of keratins K5 and K14 [3]. In response to unknown stimuli, the basal keratinocytes are triggered to differentiate terminally. They detach from the basement membrane, stop dividing, become spinous, and initiate their migration and maturation through the suprabasal layers. Concomitantly, they start to express the earliest markers of terminal differentiation, keratins K1 and K10, which are fully expressed only in the spinous and granular layers [4] [5][6]. In the granular layers, filaggrin and precursors of the cornified envelopes such, as loricrin and involucrin, as well as epidermal transgulatminase are expressed [7][8][9][10]. The final

stages of the process of differentiation occur after the dissolution of nuclei and organelles and the formation of cornified envelopes that are assembled into a metabolically inert stratum corneum.

The role of epidermal keratinocytes in defense from mechanical injury and desiccation has been appreciated for a long time, but their role in immunological defense became apparent only recently, when it was realized that keratinocytes can produce a cornucopia of growth factors, chemoattractants and cytokines [11][12][13]. Furthermore, keratinocytes express receptors for many polypeptide factors, respond to autocrine stimulation, and also respond to the signals produced by the immune system. In response to epidermal injury, keratinocytes become "activated": they produce and respond to growth factors and cytokines, become migratory and can produce components of the basement membrane. Activated keratinocytes express a specific pair of keratin proteins, distinct from the keratins in the healthy epidermis. In healthy epidermis, basal keratinocytes express K5 and K14, whereas suprabasal, differentiating keratinocytes express K1 and K10. However, in suprabasal keratinocytes of the activated epidermis, the expression of K1 and K10 is suppressed and replaced by K6 and K16. Thus, expression of K6 and K16 marks keratinocyte activation [14][15, 16]. In certain inflammatory processes, especially those characterized by

Table 1. The list of most frequently mentioned abbreviations. An attempt has been made to categorize the biologically active molecules in order to facilitate the understanding of the text for the readers less familiar with molecular biology.

Signaling polypeptides (mostly extracellular)	
IFNα, IFNβ, IFNγ	interferons
EGF	epidermal growth factor
TNFα	tumor necrosis factor a
IL-1 to IL-12	interleukins
Receptors (on the cell membrane)	
IFNR	interferon receptor
EGFR	epidermal growth factor receptor
TNFR	tumor necrosis factor a receptor
Transducing molecules (mostly in the cytoplasm)	
JAK	Janus activated kinase, transduces IFN signals
STAT (1-6)	signal transducing activator of transcription (responsive to IFNs *inter alia*)
Raf1	Ras activated factor 1
ERK	extracellular signal responsive kinase (responds to EGF *inter alia*)
MEK	mitogen activated ERK kinase (activates ERK)
TRADD	TNFR associated death domain (involved in apoptosis)
TRAF2	TNFR associated factor 2 (activates transcription)
Transcription factors (mostly in the nucleus)	
AP1	
C/EBPβ	
Elk1	
NFκB	
SAP1	

Especially relevant review articles are references [19], [23] and [40].

the infiltration of Th-1 type lymphocytes, such as psoriasis, the K17 keratin is also present [17].

The extracellular signals that induce keratinocytes to start differentiating or to become activated are at present the subject of intensive investigation. One of the initial signals for activation of keratinocytes may be the release of pre-stored IL-1. Once activated, keratinocytes synthesize additional signaling growth factors and cytokines. Several extracellular markers are specifically expressed by the activated keratinocytes. The various signaling molecules may be synergistic or antagonistic with each other. This allows the activated phenotype to be specifically modified, which can lead to different activated phenotypes. (Table 1).

Signals from the extracellular environment initiate enzymatic cascades which lead to the activation of transcription factors. Activated transcription factors then regulate gene expression by diverse mechanisms that include binding to specific DNA sequences and interaction with other transcription factors or nuclear receptors. They can also induce expression of additional regulatory factors as well as of the differentiation or activation markers. Three such pathways, IFNγ, the EGF family and TNFα/IL-1, are known to be important in keratinocyte activation and we have shown that they regulate expression of keratin genes.

The most extensively studied signaling molecules of the immune system are the interferons, IFNα, IFNβ, and IFNγ, a subset of cytokines originally described as factors that protect cells from viral infections [18][19]. IFNα and IFNβ share a cell surface receptor, whereas IFNγ binds to a different receptor and has distinct effects. Certain diseases, such as psoriasis, are associated with high levels of IFNγ in epidermis [20]. Although the role of interferons in pathologic processes has not been clearly defined, they have been used in therapeutic trials for several dermatologic diseases [21].

Activation of IFN receptors initiates a cascade of protein phosphorylation events [18][19]. The receptors interact with JAK kinases, which phosphorylate tyrosines both on the receptors, and on the STAT (for Signal Transducing Activator of Transcription) family of transcription factors (Fig. 1). The phosphorylation of STATs causes their activation and translocation to the nucleus, where STATs bind to specific DNA sites and activate transcription of nearby genes. The regulatory specificity of the cytokine signals at the cell surface is mirrored in the nucleus by the activity of specific members of the STAT family: IFNγ leads to activation of STAT1, IFNα of STAT2 and 3, IL-6 and OsM of STAT3, IL-12 of STAT3 and 4, IL-3, IL-5 and GMCSF of STAT5, while IL-4 of STAT6 [19].

Among the most extensively studied cellular receptor signaling pathways are those involving epidermal growth factor, EGF, and its receptor EGFR [22][23]. A simplified scheme of the cascade is shown in Fig. 2. Several ligands can bind to and activate EGFR including TGFα, amphiregulin, HB-EGF and herregulin. The binding of the ligand causes receptor dimerization with concomitant activation of its intracellular protein tyrosine kinase. A substrate for this kinase is the receptor itself, the two monomers phosphorylate each other. The phosphotyrosines serve as docking sites for SH2 domain containing proteins (such as Grb2 or SHC) that interact with proteins capable of activating Ras. Several growth factor receptors, via different adaptor molecules activate Ras, which makes Ras a fulcrum for signal transduction pathways (see Fig. 2). Activated Ras, in turn, activates a cascade of three protein kinases, Raf1, MEK and

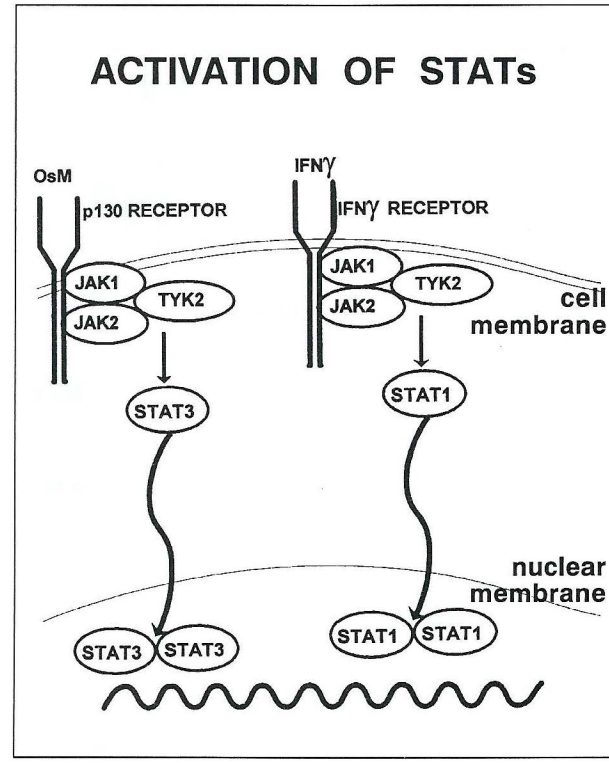

Figure 1. Activation of the JAK-STAT pathway. Binding of the ligand to the receptor causes its association with the JAK/TYK kinases, which, in turn phosporylate STATs. STATs, when phosphorylated, dimerize and translocate to the nucleus where they activate transcription.

Erk. The last one, Erk, translocates to the nucleus where it phosphorylates, and thus activates transcription factors such as Elk1and SAP1.

Successive activation of a cascade of three protein kinases is a recurrent motif in signal transduction. Stress, exemplified by osmotic shock and UV irradiation, or proinflammatory cytokines, exemplified by TNFα and IL-1, can activate two parallel cascades (see below), thus activating partially overlapping sets of transcription factors (Fig. 2). Specifically, UV irradiation primarily activates JNK, which results in activation of Jun and ATF2, whereas osmotic shock activates p38 and consequently ATF2 and Max. There can be significant crosstalk among the cascades. For example UV can activate the p38 pathway and in certain cell types, EGF can activate JNK. All three cascades are present and functional in keratinocytes.

TNFα has been discovered from two independent lines of research, first as an inducer of necrosis in some tumor cells and second as a cause of cachexia in septic animals. Subsequently it has been established that TNFα is one of the proinflammatory cytokines that induce many inflammatory effects, such as fever and shock. In response to infection or injury a wide variety of cells produce TNFα, primarily macrophages and monocytes but also epithelial cells including keratinocytes [24].

A low level of TNFα is present in the upper layers of the healthy epidermis, but its synthesis and release from keratinocytes are greatly augmented under a variety of conditions, such as allergic and

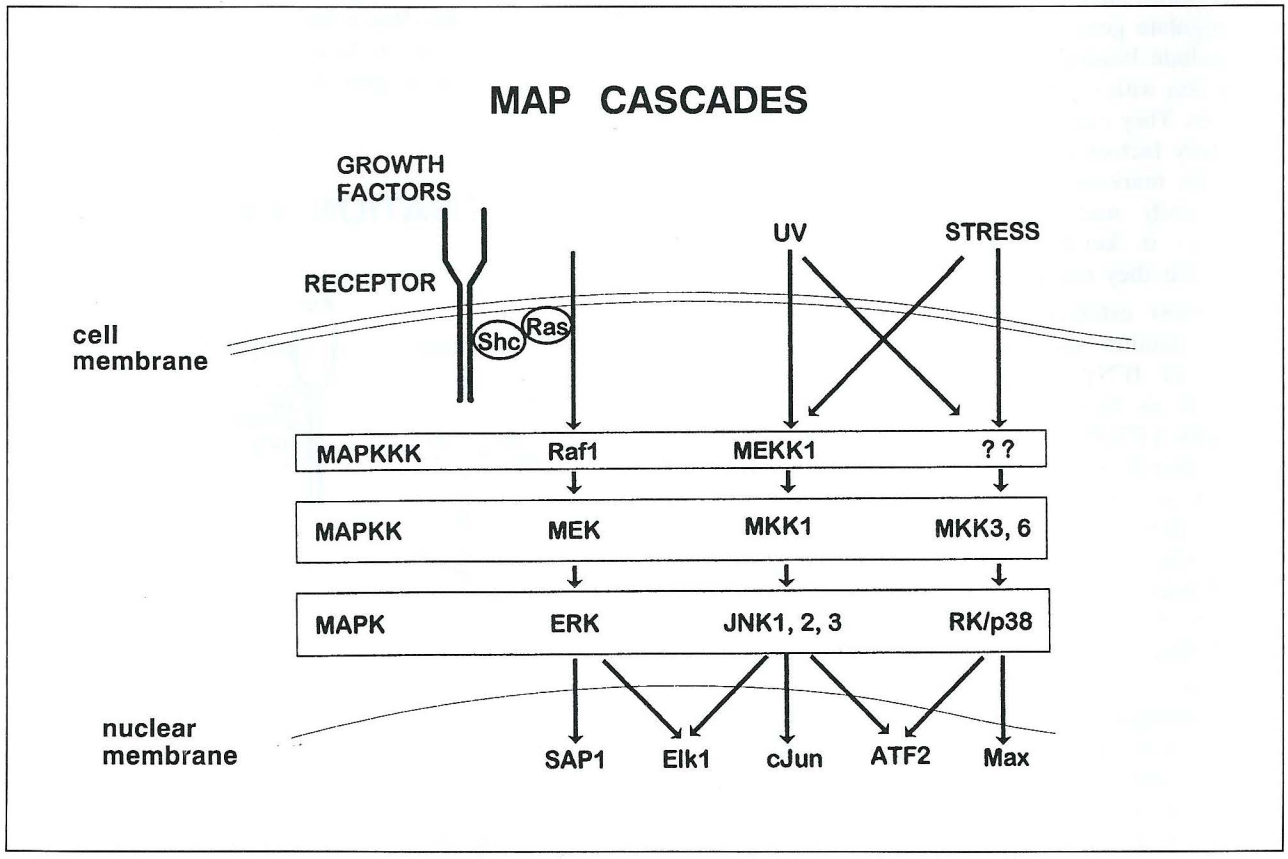

Figure 2. The MAPK signal transduction cascades. Growth factors, such as EGF, bind to their receptor activating Ras. Ras activates Raf1, which is a MAPKKK. Raf1 activates MEKs, which activate ERKs. When activated, ERKs translocate to the nucleus, where they phosphorylate and thus activate transcription factors, such as SAP1 and Elk1. Stress, such as heat, UV, or TNFα and IL-1, activate their own sets of MAPKKKs. These, in turn activate specific MAPKKs, which activate JNKs and p38. The last two MAPKs (presumably in the nucleus) phosphorylate and thus activate transcription factors shown on the bottom. While there is crosstalk at the top and the bottom of the cascades, the phosphorylation by MAPKKs and MAPKs is quite specific. Most of the initial events that sense stress signals have not been elucidated so far.

irritant contact dermatitis, infection, and UV irradiation [11]. In these pathological conditions TNFα activates immune responses by inducing production of additional signaling molecules, cytokines, growth factors, their receptors and adhesion proteins [25].

The signal transduction pathway triggered by TNFα appears to be much more complicated than the EGF-triggered one [26][27]. A currently known version of the cascade is shown in Fig. 3. There are two TNFα receptors, but keratinocytes express mainly the Type 1 receptor (55kd). There are three major intracellular effects of TNFα. One is the induction of apoptosis (Box 1 in Fig. 3). The second involves production of ceramides, which in turn act as second messengers activating arachidonic acid synthesis and regulating downstream effects (Box 2 in Fig. 3). The third signaling pathway involves proteins TRADD and TRAF2, which activate transcription factors NFκB and C/EBPβ (Box 3 in Fig. 3).

Environmental signals, such as growth factors and cytokines, modulate the activity of nuclear transcription factors, thus regulating gene expression. Modulated transcription factors fall into well characterized classes. The best studied of these are the STAT proteins, the AP1 family and the NFκB family. First discovered as mediators of interferon signaling, STATs are unusual because they can convey the signal directly from the plasma membrane into the nucleus without second messengers or cytoplasmic kinase cascade intermediates. STATs range in size from 80 to 110 kD [19]. STAT proteins are cytoplasmic in their ground state, but upon activation of appropriate

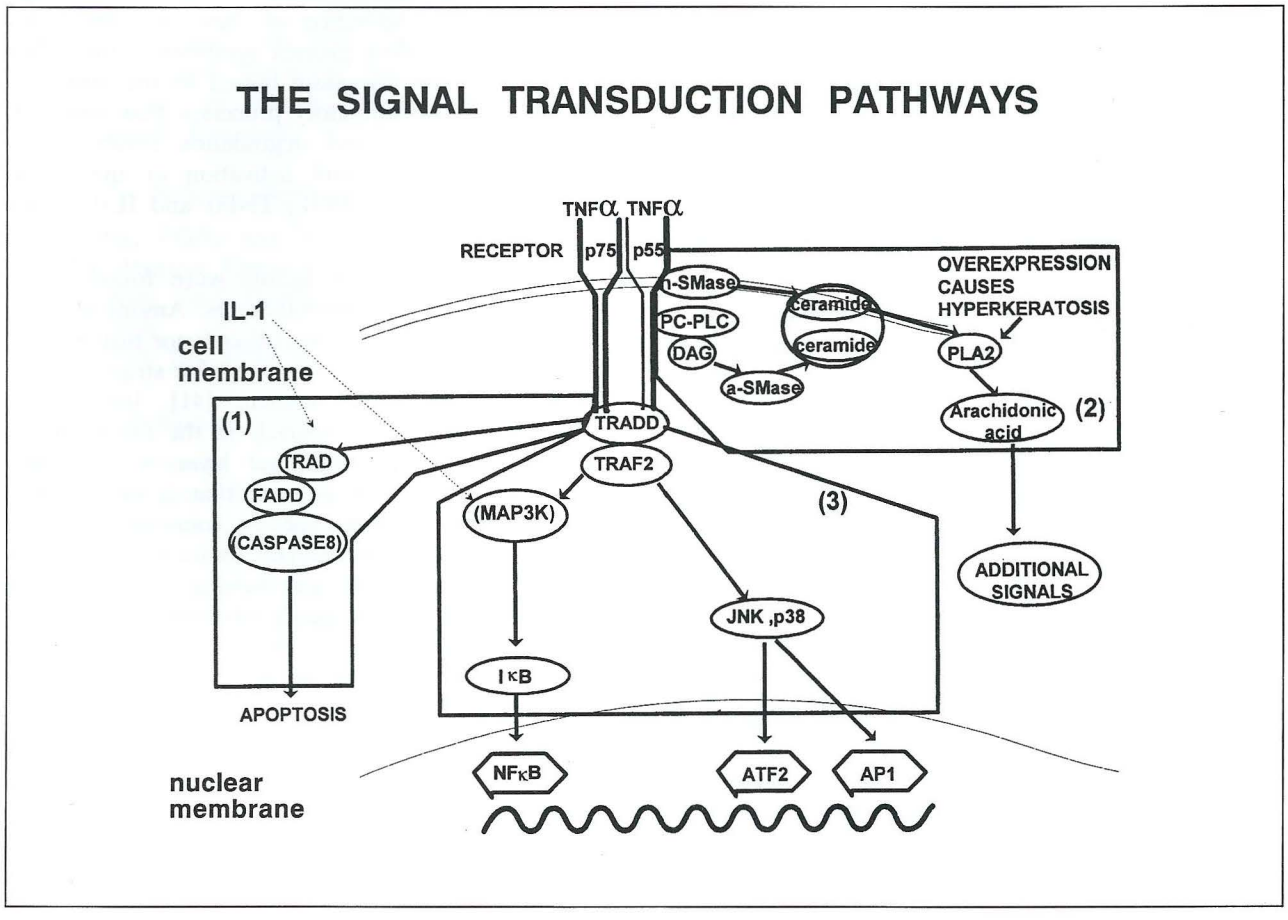

Figure 3. The TNFa signal transduction pathways. There are two receptors, although only the p55 seems to be expressed in keratinocytes. There are three principal signal transduction pathways: (1) the apoptosis pathway, (2) the ceramide pathway and (3) the TRAF2 pathway. The apoptosis pathway proceeds through "death domain" containing proteins TRAD and FADD. Through TRAK, and independently through PKC the other two MAPK pathways can get activated as well. Mechanisms by which JNK and NFkB are activated have not been fully characterized. Interestingly, the effects of IL-1 partially overlap those of TNFα.

receptors, STATs become phosphorylated and, through their SH2 domains, dimerize and translocate into the nucleus. In the nucleus STATs bind to specific DNA recognition elements and activate transcription of nearby genes.

Perhaps the most widely studied regulated transcription factors are those belonging to the AP-1 family. AP-1 is a nuclear transcription complex composed of dimers encoded by the *fos* and *jun* families of proto-oncogenes [28]. Whereas Fos proteins only heterodimerize with members of the Jun family, Jun proteins may homo- or heterodimerize with both Fos and other Jun proteins. The AP-1 activity is induced by growth factors such as serum, EGF, and TGFα, by cytokines such as IL-1 and TNFα, as well as tumor promoters such as TPA and UV light [28][29].

In the epidermis, AP-1 regulates cell growth, differentiation and transformation [30][31][32][33]. However, the expression of individual AP1 proteins

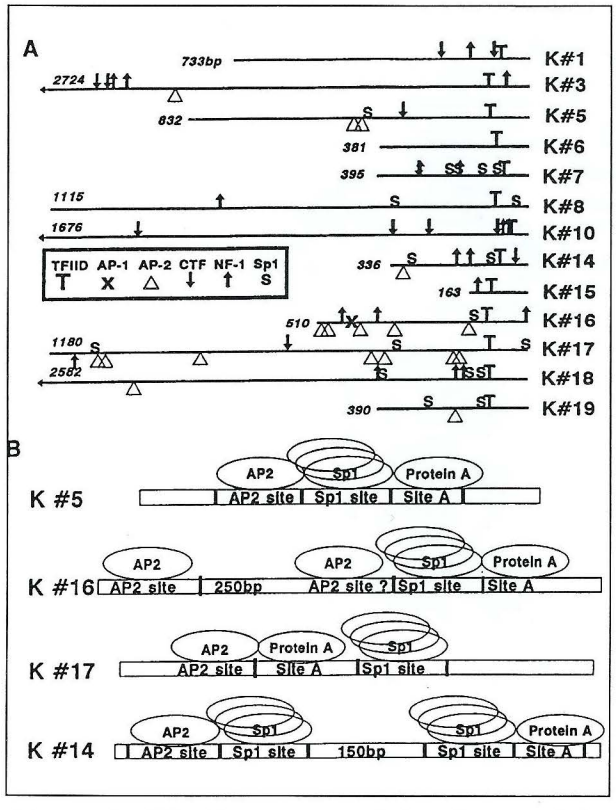

Figure 4. (A) Computer analysis of transcription factor binding sites in the sequences of the promoters of keratin genes. (B) Gel shift analysis results showing clusters of transcription factor binding sites in keratin gene promoters.

in epidermal layers is a controversial issue that awaits resolution. Certain authors find c-Fos in lower layers of the epidermis [34][30][35] while others do not find any cFos [33], which agrees with the lack of an epidermal phenotype in fos knockout mice [32]. The differing results could be explained by varied different epitopes of the antibodies used, or functional redundancy of Fos family members. Be that as it may, it is clear that the AP-1 proteins in keratinocytes can regulate the expression of differentiation markers [36][37][38] and may convey the calcium- and PKC-dependent signals [35][33]. Functional AP-1 sites have been found in many keratin genes (see below), including in the first intron of the human and murine K18 and in the K8 gene [39].

The NFκB family includes proteins p65, p50 and c/rel, which both homo- and heterodimerize amongst themselves [40]. Activation of these proteins is not dependent upon new protein synthesis, rather, they are stored in the cytoplasm bound to the inhibitory protein, I-κB. Inflammatory processes that induce I-κB phosphorylation and degradation result in the nuclear translocation and activation of the NFκB complex. Signaling by EGF, TNFα and IL-1 results in NFκB activation.

Several transcriptional factors were found to be specific for epidermal keratinocytes. Among these is Basonuclin, an unusual transcription factor that contains many pairs of Zn fingers and a serine stripe running down its alpha-helical segment [41]. Importantly, this protein is found exclusively in the nuclei of the basal and the first suprabasal layer of epidermal keratinocytes and it is possible that it plays a role in specifying basal layer specific transcription. Skn1 and Skn2 are also specifically found in skin. They are functionally distinct and belong to a family of transcription factors that specify differentiation pathways of various cell types. It is not yet known whether Skn1 and Skn2 play a similar role in epidermal differentiation.

AP2 binding sites have been found in most keratin gene promoters analyzed. Although not the sole determinant of epithelia-specific expression [42] they are essential for appropriate transcription of many keratin genes [43]. The promoters of K5, K6, K14, K16 and K17 keratin genes contain functional AP2 sites. One of the first transcription factors to be purified and cloned [44], Sp1 also belongs to a gene family. Different members of the Sp1 family can exert opposite regulation of target promoters. Interestingly, the differentiation-specific keratin genes do not have Sp1 sites in their promoters, whereas the

basal and activation-specific ones do. Sp1 interacts with other transcription factors, such as NF1 and Ets, which often have adjacent or overlapping binding sites in DNA. Sp1 protein has several trans-activation domains and can multimerize while bound to distant sites on DNA, looping out sequences between the Sp1 sites and bringing additional factors into proximity of each other [45, 46]. This, process seems important for expression of keratin genes [47]. Several NF1 binding sites have been found using computer analysis of available keratin gene promoter sequences [48]. It is important to note however that the NF1 transcription factor interacts with members of the STAT transcription factor family [49] and thus may be important for the interferon-dependent induction of K17 keratin expression.

Regulation of keratin gene expression occurs predominantly, if not exclusively at the level of transcription initiation [6]. As expected, most of the important sites for regulation are found in the upstream region although in several instances introns and even the downstream sequences contain regulatory elements.

The first cell type specific element in keratin genes was described by Blessing *et al.* [50] for the bovine K6 gene. Within a 650 bp sequence upstream from the gene exists an enhancer functional in proliferating cells of stratified epithelial origin, but nonfunctional in simple epithelial cells. This enhancer may be specific for either stratified or hyperproliferating cells, or both. It can confer enhancement of transcription to other promoters including those for K1 and K10 keratins, which are expressed only in differentiating cells and thus are not expressed without the K6 gene enhancer in cells in culture.

The upstream sequences for the following human keratin genes are known: K1, K3, K5, K6, K7, K8, K10, K14, K16, K17, K18, K19 and a hair keratin (Fig. 4). Work from our laboratory and several others has characterized many of the transcription factors and DNA sites that play important roles in regulation of keratin gene expression. All promoters have a canonical TATA box, or a variant of it. Thus, the transcription factor TFIID, which binds the TATA box, is essential for the transcription initiation of keratin genes. This is apparently the only transcription factor common to all keratin genes. In addition to the TATA box, keratin promoters contain several consensus sequences recognized by other transcriptional factors. The CAAT box is present in K1, K6, K7, K10 and K19 genes in the upstream region. NF-1 half-sites are present in K1, K3, K5, K8, K10, K14, K16 and K18 genes. Also common are the retinoic acid responsive elements, RAREs. Functional interactions have been demonstrated between AP-2 and epidermal keratin genes. The consensus sequence for AP-2 is GCCNNNGCC and similar sequences have been found in K1, K5, K6, K10, K14 and K16 genes [51]. The importance of AP-2 sites in regulating keratin gene transcription is not fully understood because disruption of the AP-2 site in the K14 gene promoters results in only a twofold reduction of transcriptional activity [51].

Keratin promoters also contain binding sites for less well known or even hitherto uncharacterized transcription factors. Transcription factors often bind to keratin genes at adjacent sites in tight clusters. Specifically, in the promoter of the K5 keratin gene, we find a complex protein binding site that binds multiple transcription factors [47]. Five different proteins, including Sp1 and AP2 independently bind to the complex. The complex is functional, as shown by point-mutagenesis and transfection assays. Interestingly, some of the same five proteins also bind to complex sites in several other keratin genes, however, while the binding proteins are the same, the sequences and the structures of the complex sites are completely different. It is as if each of these keratin gene promoters assembled a complex transcription regulatory site appropriate for the transcription factors present in epithelial cells. It is possible that such clusters confer cell type specificity to the expression of keratin genes.

Mutations currently known to cause hereditary disorders due to abnormal keratinization have all been found in genes encoding structural proteins in keratinocytes, or the enzymes that cross-link these. It is to be expected that additional mutations will be found in the regulatory circuits that govern their expression.

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