Characterization of Skn-1a/i POU Domain Factors and Linkage to Papillomavirus Gene Expression*

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Tissue-restricted POU domain transcription factors, which bind octamer or octamer-like gene sequences, play roles in cellular differentiation and the development of several organs. We have previously identified a POU domain gene, Skn-1a/i, expressed primarily in epidermis, that encodes at least two products through alternative splicing. One of these, Skn-1a, acts as a transcriptional activator, and the other, Skn-1i, contains an inhibitory domain in the NH₂ terminus, which prevents DNA-binding in vitro and transcriptional activation in vivo. We now demonstrate that when Skn-1i is expressed in eukaryotic cells it can bind to an octamer site, suggesting that in vivo cellular factors modulate the activity of the inhibitory domain to permit DNA-binding. Yet the inhibitory domain does not allow transactivation by Skn-1a or by a heterologous transactivator containing this domain in cis. Furthermore, we demonstrate that Skn-1a, Tst-1, and Oct-1 are the major octamer-binding proteins in epidermis. Since Skn-1a is primarily expressed in suprabasal cells of the epidermis, we have tested its possible role in the regulation of epidermal papillomaviruses. In transient transfection assays, Skn-1a and Tst-1 can activate the long control region of the epidermis-specific human papillomavirus 1A (HPV-1A). Consistent with these in vivo transcription data, in vitro DNA binding studies identify three octamer-like sites, which are capable of binding Skn-1a, in the HPV-1A long control region. Mutations of all three octamer-like sites prevent transactivation by Skn-1a in transient transfection assays. Taken together, these results provide evidence that Skn-1a and Tst-1 may provide a molecular link between HPV gene expression and epidermal differentiation.

During midgestation in mammals, the embryo is enclosed by a cellular bilayer composed of a basal layer of somatic ectoderm, which is covered by distinct epithelial cells referred to as periderm. While periderm is later shed, somatic ectoderm has several distinct fates in the mature organism. These fates include mammary epithelium, teeth, epidermis, and epidermal appendages such as hair, nails, and sweat glands (1). Epidermis, which forms relatively late in embryogenesis, is made of a single layer of proliferating basal keratinocytes and several layers of postmitotic suprabasal cells. The basal keratinocytes express a pair of keratins, K5 and K14, but, concomitant with departure from the basal layer, they exit the cell cycle, suppress expression of K5 and K14, and induce expression of another keratin pair, K1 and K10. As keratinocytes move outward to the surface of the skin, proteins required for formation of a cornified cell envelope are induced. These cells subsequently undergo programmed cell death to form the cornified layer of the epidermis. This differentiation process, first initiated during embryogenesis, continues throughout life with cell proliferation in the basal layer balanced by cell death and eventual shedding of the stratum corneum (2).

Human papillomaviruses (HPVs) are a family of small DNA viruses that selectively infect epithelial tissues and replicate in the nucleus of cells undergoing differentiation (3). Over 70 types of HPVs have been identified, with each type demonstrating a characteristic tissue specificity. HPV-1 and -2 primarily infect epithelial keratinocytes, causing palmoplantar warts and verruvcose warts in other regions of the skin, respectively. In contrast, HPV-16 and -18 primarily infect the genitourinary epithelium and have been implicated as the main etiologic factor in cervical cancer (3). The genomic organization of all HPVs is remarkably similar with all open reading frames located on one strand of viral DNA. The approximately 10 expressed viral genes can be divided into early (E) and late (L) genes. The early genes include E1, which is involved in replication, and E2, which regulates transcription, as well as E6 and E7, which are responsible for cellular transformation by means of interactions with p53 and Rb proteins. The late genes L1 and L2 encode for capsid proteins. Between the L2 and E6 genes there is long region in all HPVs, referred to as the long control region (LCR), which contains no open reading frames.

A characteristic feature of all these viruses is the tissue specificity of their infection and the fact that productive infections are dependent on an advanced stage of differentiation of the infected epithelial tissues. In this respect, gene expression and replication of HPV-1 and -2 correlate with expression of markers of epidermal differentiation, such as cytokeratins 1 and 10. In fact, the papillomaviruses may use the same regulatory mechanisms for transcription and replication as the cell uses for differentiation (4).

While the molecular mechanisms responsible for skin development remain unclear, they probably involve complex interactions between external regulators with convergence on nu-
clear proteins that ultimately regulate gene transcription. Recent experiments have demonstrated that the HMG domain transcription factor LEF-1 is required for development of several organs requiring inductive epithelial-mesenchymal interactions in somatic ectoderm, including teeth, mammary glands, whiskers, and hair (5, 6). A nuclear zinc finger protein, baso-nuclein, that is expressed in undifferentiated proliferating keratinocytes is likely to be a transcriptional regulator (7, 8). In addition, several transcription factors with widespread distribution have been implicated in the expression of epidermis-specific genes. These include AP-1 (9–12), AP-2 (13, 14), Mad/Max (15, 16), RARs (17, 18), HNF-4 (19), and several homeobox factors (20).

Identification of the epidermis-restricted POU domain gene Skin-1a/i (Skn-1a/i; also referred to as Epoc and Oct-11) suggested a candidate for a transcription factor involved in epidermal differentiation (21–23). This hypothesis is supported by the fact that other tissue-restricted POU domain genes have been shown to be important for cellular differentiation in other organ systems (24). Although expression has been described in the stromal cell of the thymus and in the antrum of the stomach (23), expression of the Skin-1a/i gene is essentially limited to interfollicular epidermis and cortical cells of the hair. Second, the Skin-1a/i gene is activated in somatic ectoderm at a time that corresponds with formation of epidermis and becomes primarily localized to the differentiating suprabasal layer. Finally, Skin-1a can activate transcription of the differentiation-specific K10 and SPRR2A promoters (22, 25).

Two gene products are encoded by the rat Skin-1a/i gene (22). One, Skin-1i, cloned from an anterior pituitary cDNA library, contains an NH2-terminal sequence that inhibits DNA binding in vitro and prevents transactivation in vitro. The inhibitory domain of Skin-1i is capable of transferring inhibition to unrelated DNA-binding proteins. Furthermore, despite the apparent lack of DNA-binding by Skin-1i, it can interfere with transactivation by Oct-1 and Skin-1a on octamer-containing transcription units. In the other form, Skin-1a, the inhibitory domain is replaced by a distinct sequence; this form binds octamer sites in vitro and acts as a transcriptional activator. While both forms have been found in skin by reverse transcription-polymerase chain reaction, their relative levels are unknown.

In this paper, we demonstrate that the major octamer-binding proteins in skin are Skin-1a, Oct-1, and Tst-1. We show that Skin-1i, which appears to be expressed at a relatively low level in normal skin, can bind octamer sites in eukaryotic cell extracts yet is incapable of transactivation. Both Skin-1a and Tst-1 can activate the E6 promoter of HPV-1A via octamer DNA-binding sites. Thus, the differentiation-related POU domain factors Skin-1a and Tst-1 may provide a molecular link between epidermal differentiation and efficient papillomavirus gene expression.

EXPERIMENTAL PROCEDURES

Nuclear Extract Preparation, Gel Mobility Shift Assays, and Footprint Analyses—Nuclear extracts from CV-1 cells were prepared according to the method described by Schreiber et al. (1989) (26). Cells were washed two times in phosphate-buffered saline and scraped from two 35-mm plates in 300 μl of cold buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM dithiothreitol, and protease inhibitors). After removing on ice for 5–10 min. 15 μl of 20% Nonidet P-40 was added followed by vortexing for 10 s. The lysate was centrifuged for 30 s in a microcentrifuge to separate nuclei from cytosol. After removing the cytoplasmic extract, the nuclear pellet was resuspended in 50–100 μl of buffer B (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM dithiothreitol, 400 mM NaCl, 1% Nonidet P-40, and protease inhibitors. The sample was gently rotated for 15 min at 4°C, followed by centrifugation in a microcentrifuge for 5 min. The supernatant was frozen in 10% glycerol. Nuclear extracts from neonatal mouse skin were made using modification of the same method in which 100–500 μg of tissue were dissected into small pieces followed by homogenization in buffer A using a Dounce homogenizer. Human adult skin was obtained using a dermatome set at 0.2–0.4 mm. The sample was cut into small pieces and incubated in the presence of trypsin for 1 h at 37 °C. After pipetting to dissociate the cells, the method described above was used to isolate nuclear extracts. 0.5–5 μl of nuclear extracts was used in the gel mobility shift assay as described previously (22).

The sequence of oligonucleotide binding sites used in gel mobility shift assays is as follows: 5′-AAGGGGATCCCTAGTTGCAATTGAGGATC-3′ (O+H+; octamer sequence is underlined); 5′-GATCTTGATTTA- TGAGGATCCCTAGTTGCAATTGAGGATC(T3′ (HPV sites 1 and 2; octamer-like sequences are underlined); and 5′-GATCTGATT-TGTGTTAATTTTCTGTCAGATGCAAATAAAATGTGAG-3′ (HPV site 3; octamer-like sequence is underlined). In vitro translated and bacterially expressed proteins were prepared as described previously (22). DNA-binding sites for gel mobility shifts were labeled using polynucleotide kinase. Footprint assays were performed as described previously (27).

RNA Isolation, Northern Blots, and RNase Protection Assays—Poly(A)+ RNA was isolated from mouse and rat skin as described previously (28). After gel electrophoresis, the RNA was transferred to nitrocellulose filters and hybridized to 32P-labeled DNA fragments. For Northern blots, we used a Skin-1a probe that is 350 bp long starting at the starting codon and a Skin-1i probe that is 380 bp, containing mainly 5′-untranslated sequence. These probes contain 10 bp of common sequence at the 3′-end. RNase protection assays were performed as described previously (28), using 3 μg of poly(A)+ RNA from rat neonatal skin. The cRNA probe for Skin-1a extends from the 5′-end of the Skin-1a cDNA to the HindIII site in the NH2 terminus, resulting in a protected fragment of 520 bp; the total length of the probe, including vector sequences, is 563 bp. The cRNA probe for Skin-1i extends from the 5′-end of the Skin-1i cDNA to the PstI site in the NH2 terminus, resulting in a protected fragment of 370 bp; the total length of the probe is 445 bp. An equivalent amount of probe and RNA was used for all hybridization reactions.

Generation of Antiserum—Three protein preparations, 380 Skin-1a (antisemur 813), 380 NH2 terminus of Skin-1i with the 5′-part of the POU domain (antisemur 764), and the COOH terminus of Skin-1a/i (antisemur 812), were used for immunization. Constructs encoding these proteins were expressed as glutathione S-transferase proteins in bacteria. After purification by glutathione-agarose affinity chromatography and size fractionation on SDS-polyacrylamide gels, gel slices containing these proteins were injected into rabbits according to standard protocols to generate antiserum. The Tst-1 antiserum has been previously described (29).

Plasmid Construction and Transfection Assays—Human papillomavirus 1A DNA was obtained from the American Type Culture Collection. The LCR, encompassing nucleotides 3395–4370, was isolated using PCR and cloned directionally into the luciferase reporter plasmid GeneLight (Promega), using the restriction sites EcoRI and HindIII. The sequences of the oligonucleotides used for amplification were as follows: 5′-ATATAGATCTGATATATATATATAATATATTT-3′ (5′-end) and 5′-ATATAAGCTTTGCGAGCTTATCATCTGAACTTATT-3′ (3′-end). The 5′-end of the LCR was arbitrarily assigned the number −974, and the 3′-end was assigned +1. The AvrII site at nucleotide 4314 was used to create the reporter plasmid 6′ HPV-1A LCR, which contains the minimal E6 promoter. The Bal3 restriction sites located at nucleotides 3889, 3957, and 4179, were used to create the fragments of the LCR used for cloning into reporter plasmids and for DNA-binding studies. –60 K10 luciferase contains the minimal K10 promoter upstream of luciferase in GeneLight. The following oligonucleotides were used to mutate the Skin-1a binding sites HPV site 1, HPV site 2, and HPV site 3; 5′-ATACTAATGGGAACTTACCAT-3′; 5′-GACTATTGTATGGAATCATGCC-3′; and 5′-TCACTTTATCCACATTTGCA-GGA-3′, respectively (altered nucleotides are underlined). Expression plasmids have been previously described (22, 28, 30). Transient transfection assays were performed as described previously, using 6 μg of reporter plasmid and 4 μg of expression plasmid per duplicate 35-mm tissue culture dish (22).

RESULTS

Octamer-binding Proteins in Normal Skin—To identify the octamer-binding proteins in epidermis, we isolated nuclear extracts from neonatal mouse skin. This extract was incubated with a 32P-labeled octamer site (O+H+) and subjected to non-denaturing gel electrophoresis in the gel mobility shift assay. Two major complexes are observed in skin nuclear extracts
Characterization of Skn-1a/i POU Domain Factors

(Raw text)
factor (TEF). This particular combination was selected because we have previously shown that the Skn-1i inhibitory domain can inhibit DNA-binding of TEF in vitro (22). Expression plasmids were co-transfected with the thyroid-stimulating hormone β (TSHβ) luciferase reporter plasmid. Results (the mean and range from a representative experiment) are expressed as fold activation over activity in the presence of an empty CMV vector. B, the indicated expression plasmids were co-transfected with a reporter plasmid containing two LexA binding sites upstream of the minimal prolactin promoter and luciferase. Results (mean and S.E.) are expressed as fold activation over co-transfection with an expression plasmid containing LexA DNA-binding domain alone. C, duplicate plates of CV-1 cells were transfected with the following expression plasmids: empty CMV vector (lanes 1 and 2), CMV Skn-1a (lanes 3 and 4), CMV Δ60Skn-1i (lanes 5 and 6), and CMV Skn-1i (lanes 7 and 8). Nuclear extracts were isolated, and octamer DNA binding was analyzed in the gel mobility shift assay as described in the legend to Fig. 1. The position of the Oct-1 complex is indicated with a filled arrow. F, free probe; B, bound Skn-1a/i complexes.

To define further transcriptional activation domains in the Skn-1a/i gene products, we have created a series of Skn-1a/i-LexA fusions and transfected with a reporter plasmid containing LexA DNA-binding sites into CV-1 cells (Fig. 3B). The NH2 terminus of Skn-1a shows strong transcriptional activity in this assay, while the Skn-1i NH2 terminus as well as the common POU domain and COOH terminus exhibit little or no activity. These results suggest that a major transactivation domain of Skn-1a resides in the NH2 terminus and that, in this assay, the intact NH2 terminus of Skn-1a is required for transactivation, since constructs containing either proximal or distal part of this sequence show little activity.

DNA Binding by Skn-1i—In previous studies we have noted that the Skn-1i protein produced by in vitro translation in rabbit reticulocyte lysates could not bind to octamer DNA sites and that this inhibitory effect was conferred by a short domain in the NH2 terminus (22). To test DNA binding in eukaryotic cells, we transfected CMV expression plasmids encoding variants of Skn-1a proteins into CV-1 cells. Subsequently, nuclear extracts were isolated from these cells and used to test binding to an octamer DNA site in gel mobility shift assays. Nuclear extracts from mock-transfected CV-1 cells contain one major DNA binding activity corresponding to the ubiquitous Oct-1 (Fig. 3C, lanes 1 and 2). As expected, new binding activity was found in cells transfected with Skn-1a and with Δ60 Skn-1i, a construct in which the inhibitory domain has been deleted from Skn-1i (Fig. 3C, lanes 3–6). Surprisingly, cells transfected with full-length Skn-1i showed DNA binding activity with migration between that of Skn-1a and Δ60 Skn-1i (lanes 7 and 8, Fig. 3C). These results suggest that the transcriptionally inactive Skn-1i is modified in vivo, making it capable of DNA binding.

Together, these data suggest that Skn-1i can bind DNA sites in vivo, presumably dependent upon a specific cellular machinery, but that it fails to act as a transcriptional activator, in contrast to Skn-1a, and further that inhibition can be transferred when the inhibitory domain is placed in cis with other transactivation domains.

Activation of HPV-1A Gene Expression by Skn-1a—Based on the activation properties and expression pattern of Skn-1a, we have tested whether Skn-1a can regulate the epidermis-specific HPV-1A (33, 34). The LCR containing the E6 promoter from HPV-1A was used to create a reporter construct by inserting it in front of the firefly luciferase gene (HPV-1A LCR luciferase; Fig. 4A). The isolated LCR region is 974 bp and extends from the end of the L1 gene to the beginning of the open reading frame for E6 (arbitrarily assigned the number +1). This region is the only part of the papillomavirus genome that does not contain open reading frames and is thought to contain regulatory elements in all papillomviruses. HPV-1A LCR luciferase was transiently co-transfected into CV-1 cells along with CMV expression vectors containing either full-length Skn-1a, full-length Skn-1i, Δ60 Skn-1i, Tst-1, Brn-5, or Oct-1 (Fig. 4A). CV-1 cells were selected for these studies because they lack Skn-1/i and Tst-1 POU domain factors. Of these expression plasmids, only those expressing Skn-1a, Δ60 Skn-1i, and Tst-1 activate the HPV-1A LCR luciferase plasmid. Skn-1i, Brn-5, and Oct-1 have no transcriptional effect on the HPV-1A LCR. These results indicate that Skn-1a and Tst-1 can specifically stimulate HPV-1A E6 promoter activity.

To localize the sequences in the HPV-1A LCR that mediate the Skn-1a response, we isolated four contiguous fragments from the HPV-1A LCR and cloned upstream of a minimal E6 promoter that contains 56 bp of sequence, including the TATA box (HPV-1A LCR−56; Fig. 4B). These plasmids were co-transfected with a CMV Skn-1a expression vector into CV-1 cells (Fig. 4B). The HPV-1A LCR−56 luciferase plasmid is unresponsive, whereas reporter plasmids containing either the region encompassing nucleotides −974 to −681, or nucleotides −681 to −412 are inducible by Skn-1a. CMV Skn-1a has little effect on reporter plasmids containing either the region encompassing nucleotides −974 to −681, or nucleotides −681 to −412 are inducible by Skn-1a. CMV Skn-1a has little effect on reporter plasmids containing fragments encompassing nucleotides −412 to −192 and nucleotides −192 to +1. To test whether the induction by Skn-1a depends on the E6 promoter or whether the responsiveness could transferred to a heterologous promoter, we linked the −974/−681, −681/−412, and −412/−192 fragments to the cytokeratin 10 minimal promoter (−60 K10 luciferase; Fig. 4C). These reporter plasmids were co-transfected with CMV Skn-1a into CV-1 cells (Fig. 4C). The K10 minimal promoter itself is unresponsive to CMV Skn-1a. Consistent with the previous results, only the −974/−681 and −681/−412 fragments are capable of responding to Skn-1a. Collectively, these findings suggest that Skn-1a can activate transcription of the E6 promoter through sequences in at least two regions of the HPV-1A LCR, one located between nucleotides −974 and −681 and the other located between nucleotides −681 and −412.

Binding of Skn-1a to the HPV-1A LCR—To determine whether the transcriptional regulation of the HPV-1A LCR by Skn-1a is through direct binding of Skn-1a, we performed electrophoretic gel mobility shift assays, using radioactively la-
beled fragments of the LCR and bacterially expressed Δ60Skn-1i protein. The −974/−681 (Fig. 5A, lanes 1–5), −681/−412 (lanes 6–10), −412/−192 (lanes 11–15), and −192/+1 (lanes 16–20) fragments were incubated with increasing concentrations of bacterially expressed Δ60Skn-1i protein. These reactions were then run on a nondenaturing polyacrylamide gel to separate bound from free complexes. The −974/−681 fragment binds Δ60Skn-1i with high affinity and the −681/−412 fragment with somewhat lower affinity, whereas both −412/−192 and −192/+1 fragments required high Δ60Skn-1i concentrations for DNA binding (Fig. 5A). The formation of two complexes with the −974/−681 fragment at relatively low protein concentrations suggests that this fragment contains two high affinity binding sites for Skn-1a. An antisense specific for Skn-1a causes a supershift of the complex (Fig. 5A, lane 22). We quantified the free and bound complexes shown in Fig. 5A to assess the relative affinity of Δ60Skn-1i for the fragments tested in this experiment. The relative amount of protein required for binding a half-molar amount of each fragment is an approximate guide for the relative affinity of these fragments for Δ60Skn-1i. These binding curves (Fig. 5B) confirm that the −974/−681 fragment contains the highest affinity sites, whereas the −681/−412 fragment binds with approximately 5–10-fold lower affinity. The other two fragments bind with much lower affinity.

To localize these elements more precisely, we first performed DNase I protection assays with the −974/−681 fragment and a purified Δ60Skn-1iGST fusion protein. We readily observed a footprint that corresponds to nucleotides −874 to −829 over both the + strand (data not shown) and the − strand (Fig. 5C). Inspection of the sequence of this region shows two binding sites that are perfect octamer motifs (ATGCAAAT) except for a change of 1 nucleotide (Fig. 5C). These sites are referred to as HPV sites 1 and 2 (Fig. 5E). To confirm that this region contained two binding sites, we synthesized an oligonucleotide corresponding to this region and tested it in the gel mobility shift assay (Fig. 5D). With low concentrations of Δ60Skn-1i protein, a single fast migrating complex is observed (B1). When the Δ60Skn-1i concentration is increased, a second complex (B2) with slower mobility is observed. These findings are consistent with sequential filling of the two octamer-like elements. The affinity of Δ60Skn-1i for these sites is similar to that of the affinity of Δ60Skn-1i to a classic octamer element (data not shown).

Mapping the Skn-1a binding site(s) in the −681/−412 fragment with DNase I protection assays was unsuccessful, perhaps due to the relative lower affinity of Skn-1a binding to this fragment. Therefore, we used an alternative approach in which we isolated the −681/−412 fragment and generated overlapping fragments, using different restriction endonucleases. These fragments were radioactively labeled, and their binding to Δ60Skn-1i was analyzed in the gel mobility shift assay (data not shown). Inspection of the sequence of the region mapped by this method reveals a putative binding site that contains only two nucleotide differences from a classic octamer site. Binding to this octamer-like site, referred to as HPV site 3 (Fig. 5E), was confirmed using a synthesized oligonucleotide binding site corresponding to this region (data not shown).

In summary, these results indicate that the sequences that respond transcriptionally to Skn-1a also bind Skn-1a with the highest affinity, suggesting that Skn-1a regulates the HPV-1A E6 promoter via a direct mechanism by binding to regulatory sites in the LCR.

**Mutations of Octamer-like Sequences in HPV-1A LCR Prevent Transactivation by Skn-1a**—To test the transcriptional effect of the Skn-1a binding sites directly, each site was mutated in the HPV-1A LCR luciferase plasmid. These reporter plasmids were transfected into CV-1 cells, and their ability to respond to Skn-1a was assessed by co-transfecting a control CMV vector or CMV Skn-1a (Fig. 6). While Skn-1a transactivates HPV-1A LCR luciferase, no transactivation was observed in a similar plasmid with all three octamer-like sites mutated (M123). Single mutations in site 2 (M2) or a double mutation in sites 1 and 3 (M13) had little effect on Skn-1a activation, suggesting redundancy between these sites.

Collectively, the in vitro DNA binding studies and the transient transfection analyses indicate that the Skn-1a binding sites identified in this study are both necessary and sufficient for transactivation of the HPV-1A LCR by Skn-1a.

**DISCUSSION**

POU domain factors, which bind octamer DNA sequences, have been shown to be important for cellular differentiation and development in several organs (24, 35). In this study, we demonstrate that three major octamer-binding proteins, Oct-1, Skn-1a, and Tst-1, are expressed in normal skin. Skn-1a transcripts and protein are expressed at a much lower level in
FIG. 5. Localization of Skn-1a binding sites in the HPV-1A LCR. A, The HPV-1A LCR was isolated and treated with the restriction endonuclease Rsal to generate the indicated fragments. After dephosphorylation with calf intestinal phosphatase, these fragments were \(^{32}\)P-labeled with T4 polynucleotide kinase and incubated with increasing amounts of bacterial extract containing \(\Delta 60\)Skn-1. Bound (B) and free complexes (F) were resolved in the gel mobility shift assay. To test for specificity, an antibody to the COOH terminus of Skn-1a/i was included in some reactions. The position of the supershifted complex is indicated (S). B, relative binding affinity of Skn-1a binding sites in the HPV-1A LCR. The bound and free complexes shown in Fig. 5 were excised and quantified by liquid scintillation counting. The x axis shows arbitrary units of \(\Delta 60\)Skn-1 protein, and the y axis shows the percentage of the indicated fragments that were complexed with Skn-1 protein. C, footprinting analyses of \(\Delta 60\)Skn-1i binding to the \(\sim974/681\) fragment from the HPV-1A LCR. An end-labeled \(\sim974/681\) fragment was incubated with (+) or without (−) \(\Delta 60\)Skn-1 protein, followed by a limited treatment with DNase I. The products were analyzed by denaturing electrophoresis along with Maxim-Gilbert sequencing reactions (G and G + A). The sequence of the protected region is shown on the left. Sequences (in boldface type) with high homology to octamer sites are indicated with brackets. D, binding of \(\Delta 60\)Skn-1i to tandem sites in the HPV-1A LCR. An oligonucleotide encompassing the sites identified in panel C was \(^{32}\)P-labeled and incubated with increased concentrations of Skn-1 protein. The reactions were subjected to nondenaturing gel electrophoresis. Positions of free (F) and bound (B1 and B2) complexes are indicated. Position of a complex supershifted (S) by Skn-1a/i antibody is indicated. E, alignment of the three Skn-1 binding sites in the HPV-1A LCR. The identified sites are labeled HPV #1–3. The consensus binding site for Oct-1 is shown in comparison. The 8-bp core is boxed.
normal skin than the alternatively spliced Skn-1a. We also find that Skn-1i, when expressed in eukaryotic cells, is capable of DNA binding, suggesting that in vivo its inhibitory domain acts by interfering with transactivation rather than DNA binding. Finally, we show that an epidermis-specific DNA virus, HPV-1A, contains Skn-1a-binding sites in its regulatory region that mediate transcriptional activation by Skn-1a, suggesting the possibility that Skn-1a may contribute to the tissue-specific expression of HPV-1A.

Octamer-binding Factors in Skin—The octamer (ATG-CAAAT) and related sequences are found in regulatory regions of several cellular genes. These sequences are bound by nuclear factors, collectively referred to as octamer-binding proteins. Cloning of three such factors in mammals, Oct-1, which is ubiquitously expressed, Oct-2, which is prominently expressed in B-lymphocytes, and the pituitary-specific Pit-1, revealed that all three contained a DNA-binding motif similar to that of a cell-determining factor in Caoenorhabditis elegans, unc-86 (36). The bipartite DNA-binding motif, referred to as the POU domain, is composed of a divergent homeodomain linked to another conserved region referred to as the POU-specific domain, with both domains making major groove contacts in DNA (37). Subsequently, several additional POU domain genes have been discovered in mammals and other species, many with prominent neuronal expression (38).

The interfollicular epidermis is a classic example of a tissue that is renewed by unipotent stem cells located in the basal cell layer (39). Recently, several transcription factors have been implicated in formation of both hair and teeth (5, 6), with tissue-restricted factors involved in differentiation of interfollicular epidermis remain unknown. The identification of a class II POU domain gene, Skn-1a/i, that is expressed at high levels in skin provides a candidate factor (21–23). This hypothesis is supported by the relatively restricted expression in epidermis but not other stratified epithelia, with the possible exception of stomach; expression has also been described in stromal cells of the thymus. Also consistent with a role for this gene in differentiation is the observation that in transient transfections, Skn-1a stimulates expression of a reporter that is under the control the K10 promoter (22). Interestingly, K10 is expressed in the epidermis and in stomach epithelium, the only two stratified epithelia in which Skn-1a/i expression has been described. Furthermore, Skn-1a has been shown to activate the epidermis-specific human SPRR2A gene (25). Skn-1a and Skn-1i have also been shown to inhibit the involucrin promoter, which contains an octamer DNA-binding site in its promoter. The inhibition, however, is also seen with several other POU domain factors and is independent of the involucrin octamer sites, suggesting that protein-protein interactions may be involved in this regulation (40).

In addition to Skn-1a/i, the expression of two other POU domain genes has been previously described in epidermis. One of these, Oct-1, is a class II POU domain gene that is ubiquitously expressed and has been implicated in regulation of housekeeping genes such as those encoding small nuclear RNA (41) and histone H2B (42). The other, Tst-1 (Oct-6/SCIP), is an intronless POU domain gene of class IV that exhibits predominantly neuronal and glial expression, but is also expressed in stratified epithelia throughout the body, including skin (31, 43). In transient transfection assays, Tst-1 suppressed expression of K5 and K14 reporter genes, suggesting that this factor might be involved in the restriction of K5/K14 gene expression (31). While the mRNA expression of Oct-1, Skn-1a/i, and Tst-1 has been previously described, the extent to which these protein products are expressed in epidermis was unclear.

In this study, we have shown that in Skn-1a, in addition to Oct-1, represents the major octamer binding activity in epidermal keratinocytes. Studies by Faus et al. (31) indicate that Tst-1 is the major octamer binding activity in epidermal keratinocytes. This discrepancy is likely due to the unanticipated co-migration of Skn-1a and Tst-1 by gel mobility shift and the failure of these investigators to identify the protein-DNA complexes by antisera. While the class II POU domain factors Oct-1 and Skn-1 clearly bind preferentially to classic octamer sites, other POU domain factors may bind preferentially to sites that are divergent from a classical octamer site (44). Therefore, our study does not preclude the expression of yet more such factors in epidermis. The low levels of Skn-1i transcripts coupled with the failure to observe DNA-binding complex corresponding to this factor in gel mobility shift assays suggests that Skn-1i is expressed at a very low level in normal skin.

Transcriptional Regulatory Domains in Skn-1a and Skn-1i—We previously identified a short domain in the NH2 terminus of Skn-1 that interferes with DNA binding by the POU domain in vitro (22). Intriguingly, this domain retains its activity when transferred to a distinct DNA-binding domain. Furthermore, in transient transfection assays, this domain prevents transactivation by Skn-1a/i transactivation domains, an ability we have now shown can also be transferred to a heterologous transactivation domain. The correlation between inhibition of DNA binding and lack of transactivation led to a model suggesting that Skn-1i is transcriptionally inactive because it fails to bind to DNA in vitro. The current study has led us to reevaluate this model, since we have shown that Skn-1i expressed in eukaryotic cells can bind octamer elements. It is therefore more likely that the inhibitory domain interferes with transcriptional activation function, perhaps by interacting with a transcriptional repressor(s) or by directly interfering with a transactivation domain (45). The observation that the inhibitory domain can act both on the NH2-terminal transactivation domains of Skn-1a/i and the transactivation domain of TEF suggests that protein-protein interactions may be involved. This result is also consistent with our earlier observation showing that Skn-1i can interfere with octamer-dependent transactivation by Oct-1 and Skn-1a. It is plausible that Skn-1i can occupy octamer sites, thus preventing activation by positive transactivators that bind to octamer sites. The LIM domain found in certain transcription factors may behave in an analogous fashion to the inhibitory domain of Skn-1i because it...
interferes with DNA-binding in vitro, yet DNA binding can be restored via protein-protein interactions (46, 47). However, we find that adding nuclear extracts to binding reactions containing in vitro translated Skn-1 protein does not restore binding, suggesting that the protein must be processed in the cell for binding (data not shown).

The requirement of the whole NH₂ terminus of Skn-1a for transfer of efficient transactivation to the LexA DNA binding domain suggests that several subdomains interact for full transcriptional activity similar to the transactivation domains of the related POU domain factor Oct-2 (48). Interestingly, similar to Skn-1, the Oct-2 NH₂ terminus has also been shown to contain inhibitory domains (49). Combinations of inhibitory and activation domains, each interacting with distinct or common co-regulators, may be a common regulatory mechanism for transactivators.

Skn-1a and Papillomaviruses—Expression of the viral genes is thought to be under control of an enhancer that is part of the LCR. Although at least one viral product, E2, can bind to and regulate the enhancer (50–52), the enhancer is thought to be primarily controlled by cellular trans-acting factors. While investigations of the HPV-16 and HPV-18 enhancers have implicated several ubiquitous transcription factors in regulation of these viruses, including AP-1, Sp1, and Tef-1, their differentiation- and cervix-specific activity has not been explained. Octamer DNA-binding sites have been demonstrated in the regulatory region of both HPV-16 and HPV-18. Several octamer-binding sites have been shown to bind to and regulate these virus, including Oct-1, Sp1, and Tef-1, their differentiation- and cervix-specific activity has not been explained. Octamer DNA-binding sites have been demonstrated in the regulatory region of both HPV-16 and HPV-18. Several octamer-binding sites have been shown to bind to and regulate these viruses, including AP-1, Sp1, and Tef-1, their differentiation- and cervix-specific activity has not been explained. Octamer DNA-binding sites have been demonstrated in the regulatory region of both HPV-16 and HPV-18. Several octamer-binding sites have been shown to bind to and regulate these viruses, including AP-1, Sp1, and Tef-1, their differentiation- and cervix-specific activity has not been explained.

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