

## Distinct Functional Interactions of Human Skn-1 Isoforms with Ese-1 during Keratinocyte Terminal Differentiation\*

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Among the three major POU proteins expressed in human skin, Oct-1, Tst-1/Oct-6, and Skn-1/Oct-11, only the latter induced *SPRR2A*, a marker of keratinocyte terminal differentiation. In this study, we have identified three Skn-1 isoforms, which encode proteins with various N termini, generated by alternative promoter usage. These isotypes showed distinct expression patterns in various skin samples, internal squamous epithelia, and cultured human keratinocytes. Skn-1a and Skn-1d1 bound the *SPRR2A* octamer site with comparable affinity and functioned as transcriptional activators. Skn-1d2 did not affect *SPRR2A* expression. Skn-1a, the largest protein, functionally cooperated with Ese-1/Elf-3, an epithelial-specific transcription factor, previously implicated in *SPRR2A* induction. This cooperativity, which depended on an N-terminal pointed-like domain in Skn-1a, was not found for Skn-1d1. Actually, Skn-1d1 counteracted the cooperativity between Skn-1a and Ese-1. Apparently, the human *Skn-1* locus encodes multifunctional protein isotypes, subjected to biochemical cross-talk, which are likely to play a major role in the fine-tuning of keratinocyte terminal differentiation.

The epidermis constitutes the interface between the organism and the environment and provides protection against physical, chemical, and microbial damage. The major epidermal cell type, the keratinocyte, engages in a tightly regulated process of terminal differentiation, which is essential for the protective barrier of the skin and is reflected *in vivo* by the multilayered structure of the epidermis. The innermost layer (stratum basale), connected to the dermis, comprises undifferentiated keratinocytes with a high proliferative potential. The cells committed to terminal differentiation migrate outwards into the non-dividing suprabasal layers and undergo distinct morphological and structural changes (for review, see Ref. 1). The transition from proliferating basal keratinocytes to terminally differentiated cells is accompanied by a significant alteration in the gene expression program. The repression of genes required for cellular growth contrasts with the induction of genes related to cell death and cornification. Aberrations in

this tightly choreographed process will affect the expression of epidermal structural proteins, such as those involved in the formation of cytoskeleton, desmosomes, and cornified cell envelopes. As a matter of fact many genetic and acquired human dermatoses have been linked to mutations or aberrant expression of these proteins (2, 3).

Whereas the importance of structural proteins in safeguarding the integrity of epidermis and internal squamous epithelia is becoming well understood, little is yet known about the regulatory processes that are involved. Although ubiquitous transcription factors contribute to keratinocyte-specific gene expression (4–7), the complex balance between proliferation, stratification, and cornification is likely to be coordinated by cell type-specific proteins. Good candidates for such a function are the POU domain transcription factors, a family of more than 40 homeodomain-containing proteins involved in cell differentiation and tissue specification (8, 9). The characteristic POU domain consists of two conserved regions, a POU-specific domain and a POU homeodomain, connected by a hypervariable linker region. The entire POU domain is required for DNA binding. The octamer 5'-ATGCAAAT-3' is the most frequent target for POU domain proteins (10).

Major POU domain factors in skin are Oct-1, Tst-1/Oct-6, and Skn-1/Oct-11 (11). The ubiquitous Oct-1 is expressed in both proliferating and differentiating epidermal keratinocytes, whereas Oct-6 and Skn-1 are primarily expressed in suprabasal layers. Skn-1 is selectively expressed in the epidermis (12–15). *In vivo* ablation of murine *Skn-1* did not reveal a specific function for this gene, mainly due to redundancy with Oct-6 (12). Recently however, the use of *in vitro* raft cultures disclosed a regulatory role of Skn-1 in keratinocyte proliferation and differentiation (16).

A further degree of regulatory complexity is due to the fact that at least several POU genes give rise to various isoforms, with specific functional properties and expression patterns (*e.g.* Oct-1, Oct-2, Brn-3, and Pit-1) (8). Also, the rat *Skn-1* gene was shown to generate two functionally distinct transcripts, *Skn-1a* and *Skn-1i* (13). Here we show that the human homologue expresses three isoforms that differentially affected the expression of the *SPRR2A* cornified envelope precursor gene, a marker of keratinocyte terminal differentiation, whose regulation has been extensively studied (5, 17). This isotype-specific selectivity in *SPRR2A* regulation, which varied from activation to repression, depended on the differential interaction of the Skn-1 isoforms with the epithelium-specific Ets factor Ese-1 (18).

### EXPERIMENTAL PROCEDURES

*Screening of a Keratinocyte cDNA Library*—A human keratinocyte cDNA library constructed in Lambda ZAP II (Stratagene) (19) was screened with probes from the POU domains of Oct-1 and Oct-2 (20, 21) at a stringent hybridization temperature. This yielded among others two independent Skn-1 clones. Plasmid DNA was isolated by *in vivo*

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excision with Exassist M13 helper phage (Stratagene).

**5'-Rapid Amplification of cDNA Ends (RACE)**<sup>1</sup>—5'-RACE was performed on poly(A) RNA isolated from cultured normal human keratinocytes essentially according to a previously described method (22). Briefly, cDNA synthesized from 1  $\mu$ g of poly(A) RNA, primed with a 5'-biotinylated antisense oligonucleotide specific for Skn-1 (5'-biotin-GAAACCTCTTCTCCAGAGTCAGGCGG), was purified on Dynabeads coated with streptavidin (Dyna) and ligated to a 5'-phosphorylated and 3' 3-amino-2-propanol-ether-blocked RACE-anchor (5'-phosphate-GC-GGCCGCGTCTGACTGGGAAAACCCOCH<sub>2</sub>CHOHCH<sub>2</sub>NH<sub>2</sub>). PCR primed with various reverse Skn-1 primers (1, ACCAAATACCTCACT-GAGGCTGGGGTAGGAG; 2, AACCCCGCAGCCCCACATCTCCCTGT; 3, GAGGAGACCGCTTTGTTGCTGTGGA; positions in GenBank<sup>TM</sup> accession number AF133895 are 814, 631, and 394, respectively) and a RACE primer complementary to the anchor (GGGTTTTCCAGTCAC-GACGCGGC) was performed with a 2.5:1 mixture of *Pwo* polymerase (Roche Applied Science) and *Taq* polymerase (HT Biotechnology Ltd.) for 40 cycles (20 min at 94 °C, 30 min at 50–60 °C, 2 min at 72 °C). Fragments were cloned in pBluescript II SK(-) (Stratagene).

**Inverse PCR**—Genomic DNA was isolated from either simian COS-1 cells or mouse 3T3 fibroblasts by proteinase K digestion and phenol extraction. One  $\mu$ g of DNA was digested with either *Bst*YI or *Sau*3AI and ligated with T4 DNA ligase (Amersham Biosciences). Ligated DNA was used in a PCR reaction with primers designed to contain restriction sites at the 5' end to facilitate subsequent cloning (simian sense primer, CGAATTTCCACAGACTGG GCCGGGACT; mouse sense primer, AGAATTTCCACAGACAGGGCCTGGCCT, derived from the mouse cDNA sequence (GenBank<sup>TM</sup> accession number Z18537); common antisense primer, AGAAAGCTTTTGTGCTGTGGAAAGG).

**Semiquantitative RT-PCR and RNA Blotting**—Trizol reagent (Invitrogen) was used to isolate total epidermal RNA from tissue obtained either after breast reduction or circumcision. Total RNA (200 ng) was reverse-transcribed with Super-RT (SphaeroQ) and random hexamers (Amersham Biosciences). Semiquantitative PCR was performed according to a previously published procedure (23) but using AmpliTaq Gold (Roche Applied Science). The following isotype-specific Skn-1 primers were used: Skn-1a: sense, CACAGATATCAAGATGAGTG, antisense, TCTGAGATAGCAGGAAGT; Skn-1d1: sense, GTTGTAGCACATGT-GTTTCA, antisense, GAAACCTCTTCTCCAGAGTCAG; Skn-1d2: sense, TCACCTTAGAGGGAGGAGA, antisense, CAGCCGGGAGTTG-TAGAC. This analysis resulted in products of 330, 581, and 590 base pairs for Skn-1a, Skn-1d1, and Skn-1d2, respectively. Other primers were: SPRR2A: sense, TGGTACCTGAGCATCGATCTGCC, antisense, CCAAATATCCTTATCTTTCTTGG (23); ESE-1: sense, CTGAGCAAAGAGTACTGGGACTGTC, antisense, CCATAGTTGGGCCACAGCCTC-GGAGC. RT-PCR conditions for GAPDH were described previously (24). All primers bridged introns, thus allowing a control for DNA contamination. PCR products for the various Skn-1 isoforms were analyzed on a blot with a probe covering most of the cDNA (generated with the Skn-1a sense and the Skn-1d2 antisense primers). Sequence analysis was used to verify the identity of the various PCR products obtained. SPRR2A RT-PCR products were identified with an SPRR2 cDNA probe (25). ESE-1 and GAPDH products were detected by ethidium bromide staining. RNA dot-blots were probed with a 511-bp *Kpn*I/*Eco*RV fragment (3'-UTR) of Skn-1 (GenBank<sup>TM</sup> accession number AF133895).

**In Situ Hybridization**—Experiments were carried out as described previously (24). Digoxigenin-labeled (Roche Applied Science) sense and antisense RNA probes were generated using the 511-bp *Kpn*I/*Eco*RV fragment (3'-UTR) of Skn-1 described above, a 680-bp fragment of SPRR2 (26), and a 475-bp *Xho*I/*Bgl*II fragment (3'-UTR) of ESE-1 (GenBank<sup>TM</sup> accession number U73844).

**Expression Plasmids**—Protein expression plasmids were constructed by introducing the *Skn-1* coding sequences in the *Hind*III and *Eco*RI sites of the T7 expression plasmid pT7-2. Plasmids encoding the whole open reading frame were generated for *Skn-1a* (pPOU117, from exon 1–13), *Skn-1d1* (pPOU123, from codon A in intron 5 to exon 13), and *Skn-1d2* (pPOU121, exon 8–13). For *Skn-1d1*, 3 mutants were constructed either by site-directed mutagenesis (point mutations in pPOU124 and pPOU118) or by PCR (pPOU137). Proteins encoded by these plasmids were synthesized in a coupled *in vitro* transcription-translation system (TnT reticulocyte lysate, Promega) in the presence of

[<sup>35</sup>S]methionine (Amersham Biosciences). Full-length *Ets-2*, *Ese-1*, and *Oct-2* cDNAs were isolated by screening the above-mentioned human keratinocyte cDNA library either with *Ets*-specific (27) or *Oct-1/2* POU domain probes. For transfection the different cDNAs were cloned into the RSV-H20 expression plasmid (28). Expression plasmids for *Oct-1* and *Oct-6* were gifts of Dr. W. Herr (Cold Spring Harbor) and Dr. D. Meijer (Erasmus University Rotterdam), respectively.

**Electrophoretic Mobility Shift Assay**—Electrophoretic mobility shift assays were performed as described in Fischer *et al.* (5). The SPRR2A octamer oligonucleotide (GGATAAATTTGCATCTGGCT) was labeled with T4 polynucleotide kinase, purified by denaturing gel electrophoresis and reverse-phase chromatography, and subsequently annealed to the unlabeled complementary strand. In each reaction 2  $\mu$ l of programmed reticulocyte lysate and 20 fmol of labeled oligonucleotide duplex were used.

**Cell Culture, Transient Transfections, CAT and Luciferase Assays**—HaCaT cells were grown in DMEM with 10% bovine calf serum (HyClone). Confluent cultures were transfected by incubating 5 cm culture dishes for 2 h with 5  $\mu$ g of reporter plasmid (CAT or luciferase), 2.0  $\mu$ g of Rous sarcoma virus expression plasmids (including compensating amounts of empty Rous sarcoma virus vector), and 40  $\mu$ g of *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium salts transfection reagent. Monolayers were washed with phosphate-buffered saline and incubated for 24 h in culture medium. CAT assays were performed as described (5). Luciferase activity was measured with the luciferase assay system (Promega) essentially as previously described (28). All transfections were performed at least in triplicate.

The SPRR2A minimal promoter-driven CAT plasmid pSG55 has been previously described (5). Luciferase plasmids were constructed in pGL3 (Promega) and contained the following SPRR2A promoter inserts, all derived from previously described plasmids (5): pSG350-wt (minimal promoter), pSG55; pSG390-*Ets* mutant, pSG212; pSG527-octamer mutant, pSG185.

## RESULTS

**Structural Characterization and Organization of Skn-1 Isoforms**—Searching the human genome data base at NCBI with the previously described human *Skn-1a* cDNA (GenBank<sup>TM</sup> accession number AF133895) disclosed one sequence (GenBank<sup>TM</sup> accession number AP 001150), encompassing the complete human gene. The 2868-bp cDNA sequence comprises 13 exons and extends over a 70-kilobase genomic region (Fig. 1A). The characteristic POU domain is encoded by exons 7–10.

The screening of a human keratinocyte cDNA library with a POU domain probe and 5' RACE (see "Experimental Procedures") identified three Skn-1 isoforms, namely the previously described Skn-1a (29) and two novel variants, Skn-1d1 and Skn-1d2. Comparison with the genomic sequence revealed that the 5' end of *Skn-1d1* and *Skn-1d2* corresponded to sequences in introns 5 and 7, respectively (Fig. 1A). The absence of other introns in these transcripts confirmed the mRNA origin of both clones.

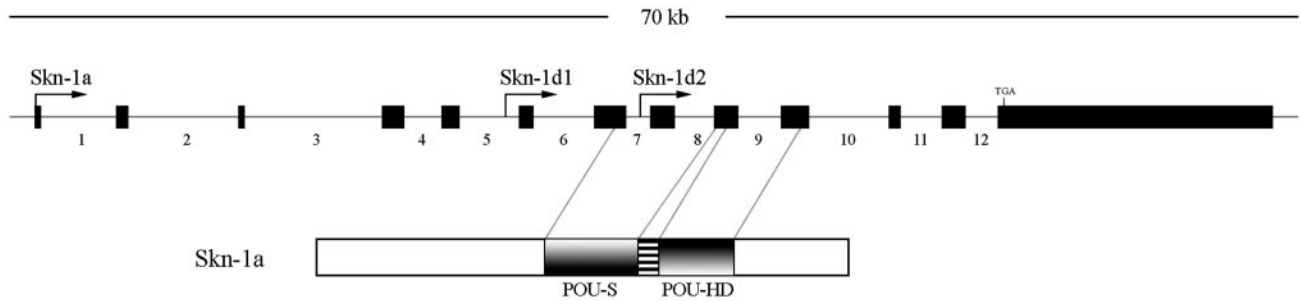
The human *Skn-1d1* transcript is homologous to the *Skn-1i* variant of rat (13) and is compared in Fig. 1B with the corresponding simian and rodent sequences. The translation initiation codon previously identified in the rat (codon 1) is not present in the human sequence. Both primate genes contain an AUG codon (codon A), which is in-frame with the *Skn-1a* coding sequence. However, this start codon is not likely to be functional since it is followed by a termination codon UAG (codon B) that is not present in rodents. A putative low affinity initiation codon CUG for *Skn-1d1* is found at position 85 in the beginning of exon 6 (codon C) (Fig. 1B).

The sequence of the *Skn-1d2* transcript, which initiates in intron 7, revealed 3 intron-encoded AUG codons in-frame with the POU domain sequence but followed by a termination codon at position 154 (Fig. 1C). Potential start codons for open reading frames are found in exon 8 (codon D and/or codon E).

**DNA Binding Activity of the hSkn-1 Variants**—The integrity of the Skn-1 expression plasmids and their coding potential were verified by producing proteins *in vitro* with the TNT reticulocyte lysate system (Promega) (Fig. 2B). Skn-1a (pPOU-

<sup>1</sup> The abbreviations used are: RACE, rapid amplification of cDNA ends; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UTR, untranslated region; CAT, chloramphenicol acetyltransferase.

A



B Skn-1d1

	Human	1	g	T	G	g	g	t	t	g	c	c	a	a	a	*	-	t	t	g	t	t	a	a	a	a	a	c	c	a	a	a	c	c	c	c	c	a	a	A	T	G	t
	Simian	1	A	T	G	g	g	t	t	t	c	c	g	a	a	a	a	-	t	t	g	t	t	a	a	a	a	a	c	c	c	c	c	c	c	c	c	c	c	A	T	G	t
	Rat	1	A	T	G	g	g	t	t	t	c	c	g	a	a	a	a	-	t	t	g	t	t	a	a	a	a	a	c	c	c	c	c	c	c	c	c	c	c	A	T	G	t
	Mouse	1	A	T	G	g	g	t	t	t	c	c	g	a	a	a	a	-	t	t	g	t	t	a	a	a	a	a	c	c	c	c	c	c	c	c	c	c	c	A	T	G	t

	Human	26	g	t	t	t	c	a	a	a	A	T	A	G	c	c	t	g	g	a	t	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	Simian	26	c	t	t	t	c	a	a	a	A	T	A	G	c	c	c	t	g	g	a	t	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Rat	23	c	t	t	t	c	a	a	a	T	A	G	c	c	c	c	t	g	g	a	t	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Mouse	24	c	t	t	t	c	a	a	a	t	g	g	c	c	c	c	t	g	g	a	t	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	Human	44	-	-	-	-	-	-	-	-	-	-	-	-	t	t	t	a	t	t	t	g	t	c	c	t	t	c	e
	Simian	44	-	-	-	-	-	-	-	-	-	-	-	-	t	t	t	a <td>a</td> <td>t</td> <td>t</td> <td>t</td> <td>g</td> <td>t</td> <td>c</td> <td>c</td> <td>t</td> <td>t</td> <td>e</td>	a	t	t	t	g	t	c	c	t	t	e
	Rat	43	t	g	t	t	g	t	t	g	t	t	g	t	t	t	t	g	t	t	t	t	g	t	c	c	t	t	e
	Mouse	48	t	g	t	t	g	t	t	g	t	t	g	t	t	t	g	t	t	t	t	g	t	c	c	t	t	e	

	Human	55	g	t	t	c	c	t	t	g	t	c	c	c	c	c	c	c	c	t	t	t	c	a	c	c	-	-
	Simian	55	g	t	t	c	c	t	t	t	g	t	c	c	c	c	c	c	c	c	t	t	t	c	a	c	c	-
	Rat	68	g	t	t	c	c	t	t	t	g	t	c	c	c	c	c	c	c	c	t	t	t	c	a	c	c	-
	Mouse	73	g	t	t	c	c	t	t	t	g	t	c	c	c	c	c	c	c	c	t	t	t	c	a	c	c	-

	Human	80	c	a	a	g	G	T	C	T	G	C	A	G	C	C	C	A	A	A	T	C	T	T	C	C	T	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	
	Simian	80	c	a	a	g	G	T	C	T	G	C	A	G	C	C	C	A	A	A	T	C	T	T	C	C	T	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Rat	92	c	a	a	g	G	T	C	T	G	C	A	G	C	C	C	G	A	A	T	C	T	T	C	C	T	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Mouse	98	c	a	a	g	G	T	C	T	G	C	A	G	C	C	C	G	A	A	T	C	T	T	C	C	T	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-

C Skn-1d2

1	accttagagggaggagacttggaaag	
27	gagccgagcatgggtgagcactcaaa	
53	gattcaggctggctgtgtgatc <b>ATG</b>	
79	ctaggaccatgcagg <b>ATG</b> tgggcaaa	
105	caagaggaggaggctgcctctc <b>ATG</b> t	
131	tcctcccctcggtccccacgct <b>TAG</b>	
157	<u>GGAGATGTGGGGCTGGCC<b>ATG</b>GGAAA</u>	codon D
183	<u>GCTGTATGGCAACGACTTCAGCCAGA</u>	E X O N
209	<u>CCACCATCTCACGATTTGAGGCCCTC</u>	
235	<u>AACCTGAGCTTCAAGAAC<b>ATG</b>TGCAA</u>	

FIG. 1. A, genomic organization of the human Skn-1 gene. The transcription start sites for Skn-1a, Skn-1d1, and Skn-1d2 are indicated by arrows. The POU-specific domain (*POU-S*) and the POU-homeodomain (*POU-HD*) of Skn-1a are represented. Exons are boxed, and introns are numbered. kb, kilobases. B, part of human intron 5 is compared with the corresponding sequence from simian, rat (GenBank<sup>TM</sup> accession number L23863), and mouse. Simian and murine sequences were obtained from inverse PCR on Cos-1 and 3T3 cells, respectively ("Experimental Procedures"). An asterisk indicates the start of the longest 5'-RACE clone for Skn-1d1. Bases shown in lowercase correspond to intronic sequences; various initiation and termination codons that are discussed in "Results," and the 3' splice-site of intron 5 is highlighted. C, sequence of the longest 5'-RACE clone for Skn-1d2. Potential initiation and termination codons are indicated. Intron sequences are shown in lowercase. The sequence of exon 8 is underlined. The 3' splice site is represented in bold.

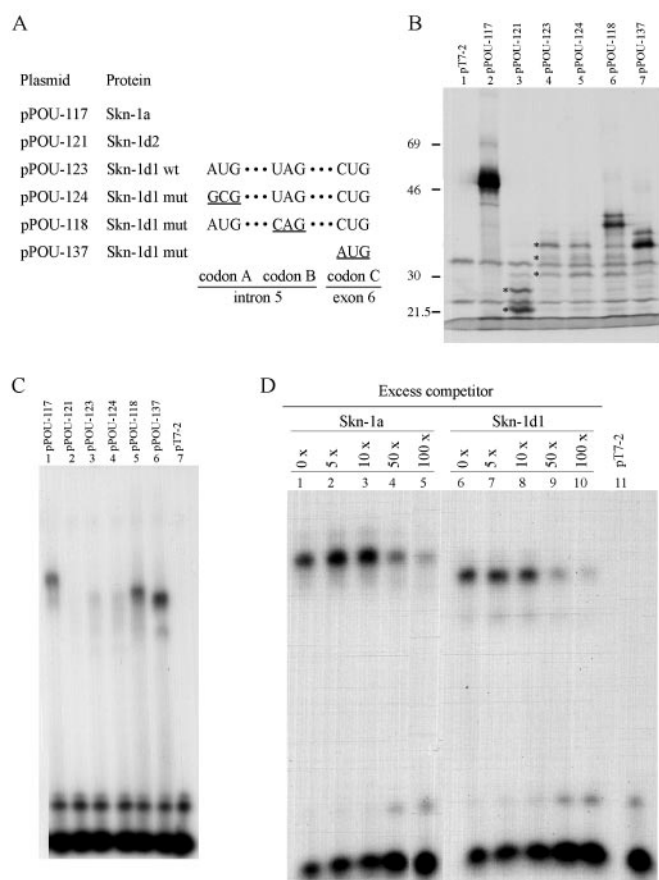
117) migrated at the predicted molecular mass (47.5 kDa) and bound to the *SPRR2A* octamer site (Fig. 2C). The Skn-1d2 isoform (pPOU-121) generated two different products (Fig. 2B). The slow migrating product has an apparent molecular mass of 25 kDa, which is in accordance with a protein initiating at codon D in the POU-specific domain (Fig. 1C). The 21.5-kDa product corresponds to a protein starting at initiation codon E. None of these Skn-1d2 isoforms is capable of binding to the *SPRR2A* octamer site (Fig. 2C), which is likely due to the partial deletion of the POU-specific domain (Fig. 1A).

The wild type *Skn-1d1* transcript (plasmid pPOU-123) codes for a major polypeptide of 35 kDa and two minor products of 33 and 31 kDa (Fig. 2B). To investigate translational initiation of Skn-1d1 proteins more precisely, constructs with mutations in codons A, B, or C (Fig. 1B and 2A) were generated. Fig. 2B shows that translation of the major Skn-1d1 polypeptide most likely initiates at codon C (a weak CUG initiation codon). Indeed, pPOU-137, in which this codon was changed into an efficient AUG initiator, yielded high levels of a product of 35 kDa, identical to the largest polypeptide from the wild type transcript. This cDNA was used in transfection experiments. The minor 33- and 31-kDa proteins were not investigated further.

Electrophoretic mobility shift assays were performed to compare the binding affinity of Skn-1a (pPOU-117) and Skn-1d1 (pPOU-137) to the *SPRR2A* octamer site. Labeled double-stranded *SPRR2A* octamer oligonucleotide was incubated with reticulocyte lysate programmed with either Skn-1a or Skn-1d1. As shown in Fig. 2D, competition with increasing amounts of unlabeled binding site revealed that both Skn-1 variants bound the *SPRR2A* octamer site with similar affinities.

*Transactivation Potential of Skn-1 Isoforms*—In Fig. 3A, the *SPRR2A* proximal promoter, fused to the CAT reporter and encompassing all cis-elements necessary for expression during keratinocyte terminal differentiation (5), was transiently transfected into HaCaT cells, which contain low levels of endogenous POU proteins (16). We first evaluated the transactivation potential of Oct-1, Oct-6/Tst-1, and Skn-1a, the major POU domain proteins expressed in skin (11), and of Oct-2, a lymphoid-specific transcription factor (21), also expressed in cultured human keratinocytes (12).<sup>2</sup> Oct-1 and Oct-2 did not affect base-line expression of *SPRR2A*, Oct-6 repressed promoter activity by ~70%, and Skn-1a was the only POU domain

<sup>2</sup> A. Cabral, D. F. Fischer, W. P. Vermeij, and C. Backendorf, unpublished observation.

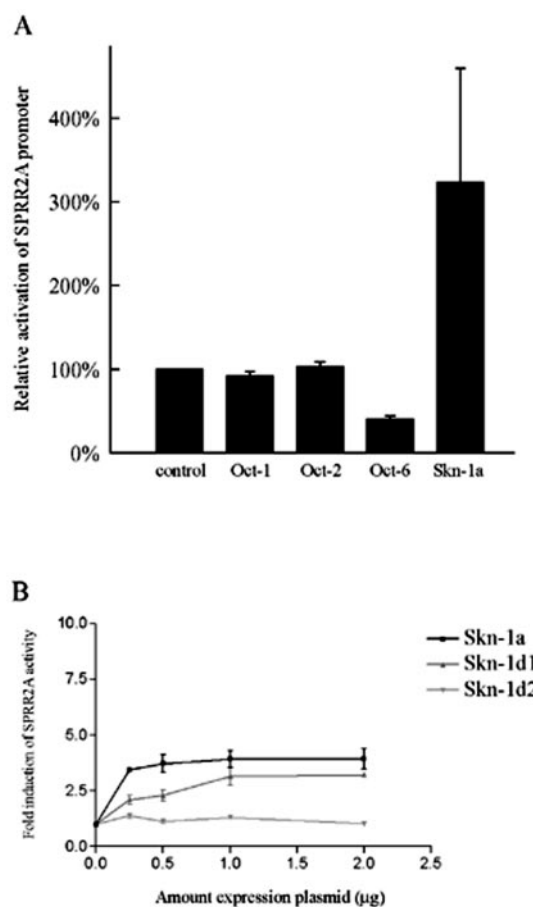


**FIG. 2. DNA binding activity of *in vitro* synthesized Skn-1 isoforms.** A, plasmids used for *in vitro* transcription-translation. The various mutations in *Skn-1d1* are indicated. Nomenclature of codons A, B, and C is according to Fig. 1B. B, SDS-PAGE analysis of *in vitro* synthesized Skn-1 proteins. Reticulocyte lysates were programmed with the indicated *Skn-1* plasmid or with a control plasmid (pT7-2, lane 1). Molecular mass markers (Amersham Biosciences) are indicated in the margin (in kDa). Skn-1d1- and Skn-1d2-specific products are marked with asterisks. C, electrophoretic mobility shift assay of Skn-1-programmed lysates (lanes 1–6) or un-programmed lysate (lane 7) with the *SPRR2A* octamer site. D, affinity of Skn-1a or Skn-1d1 for octamer binding; reticulocyte lysate containing identical amounts of either Skn-1a or Skn-1d1 protein were incubated with 20 fmol of the <sup>32</sup>P-labeled *SPRR2A* octamer site and competed with the indicated molar excess of unlabeled binding site.

protein tested that mediated gene activation (3–4-fold induction, Fig. 3A).

To investigate the relative contribution of the individual Skn-1 isoforms in the regulation of the *SPRR2A* gene, the transactivation potential of the three variants was determined by transfecting increasing amounts of isotype-specific expression plasmids into HaCaT cells, programmed with an *SPRR2A*-luciferase construct. Both Skn-1a and Skn-1d1 up-regulated the *SPRR2A* promoter by 3–4-fold. The saturation kinetics were, however, different because lower amounts of Skn-1a were needed to reach a plateau, indicating that Skn-1a can transactivate the *SPRR2A* promoter more efficiently than Skn-1d1 (Fig. 3B). Skn-1d2 had no effect on *SPRR2A* promoter activity, even at higher doses. Collectively, these results indicate that among several POU domain proteins only Skn-1a and Skn-1d1 are able to activate the *SPRR2A* promoter in an *in vitro* transient transfection experiment. Hence, it was important to investigate whether a similar direct relation existed also *in vivo* between Skn-1a/d1 and *SPRR2A* expression.

**Correlation between *SPRR2A* and *Skn-1a* Expression *in Vivo***—Initially, a panel of 50 different RNA samples from var-



**FIG. 3. Effect of various POU proteins on *SPRR2A* promoter activity.** A, the *SPRR2A*-CAT reporter construct (pSG55) was either transfected alone or together with expression plasmids for Oct-1, Oct-2, Oct-6, and Skn-1a into HaCaT keratinocytes as described under “Experimental Procedures.” CAT activity was determined 24 h after transfection as previously described (5) and was related to the basal activity of pSG55. B, increasing amounts of Skn-1a, Skn-1d1, and Skn-1d2 expression plasmids were co-transfected with the *SPRR2A*-luciferase reporter construct (pSG350), and luciferase activity was determined 24 h later essentially as previously described (28).

ious human tissues was analyzed with a *Skn-1*-specific probe (3'-UTR), which detects all isoforms. *Skn-1* expression was restricted to a few stratified squamous epithelia including epidermis, cervix, and foreskin (results not shown). To compare the expression pattern of the three human *Skn-1* variants with *SPRR2A* expression, semiquantitative RT-PCR with isotype-specific primer sets was performed on RNA isolated from either total skin, epidermis, foreskin, cervix, or cultured primary keratinocytes. RNA from uterus, which does not contain *SPRR2A* transcripts (23), was used as a negative control. The results in Fig. 4A show that *Skn-1a* was expressed in all squamous epithelia (lanes 1–6 and 8) and in cultured keratinocytes (lane 7), although at different levels. Expression of *Skn-1d1* was more heterogeneous, as it was not detected in the skin and cervix samples from lanes 1 and 8, respectively. Relatively high expression levels of *Skn-1d1* (already visible after 25 cycles) were found in the epidermal RNAs from lanes 2, 3, and 5. *Skn-1d2* expression was, in general, similar to the one of *Skn-1a*. The absence of *Skn-1* transcripts in uterus correlated well with the absence of *SPRR2A* expression. However, no clear correlation was found in the other samples between the expression level of one of the *Skn-1* isotypes and *SPRR2A* expression, indicating the involvement of other transcription factors. Indeed, previous work from our laboratory has shown that expression of *SPRR2A* relied on interdependent regulatory promoter ele-

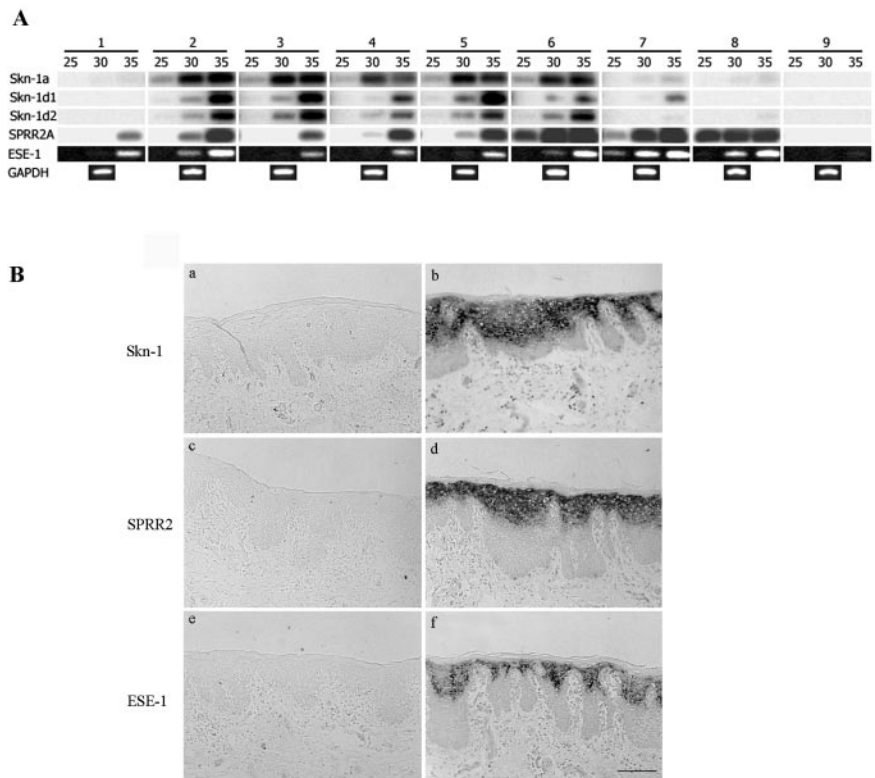


FIG. 4. A, semiquantitative RT-PCR analysis of Skn-1, Ese-1, SPRR2A, and GAPDH expression in various human tissues and cells. RNA isolated from total skin (lane 1), isolated epidermis (lanes 2–5), foreskin (lane 6), cultured primary keratinocytes (lane 7), cervix (lane 8), and uterus (lane 9) were analyzed with SPRR2A, Ese-1, GAPDH, and isoform-specific Skn-1 primers. PCR was performed with 25, 30, and 35 cycles, and products were either detected with gene-specific probes (Skn-1 isoforms and SPRR2A) or by direct ethidium bromide staining (Ese-1 and GAPDH). For GAPDH only the 30-cycle product is shown. B, *in situ* hybridization performed with digoxigenin-labeled Skn-1-, SPRR2-, and Ese-1-specific RNA probes. A foreskin section was analyzed with sense (a, c, e) and antisense (b, d, f) probes. Bar, 100  $\mu$ m.

ments, recognized by various classes of transcription factors (5). Consequently, we have also monitored the expression of the epithelial-specific Ets factor Ese-1, previously implicated in SPRR regulation (18, 27). Expression of this gene was found in all samples, including uterus (although at low levels). The highest levels were found in the epidermal sample of lane 2, in cultured keratinocytes (lane 7), and in cervix (lane 8). Again, no strict correlation was found between the levels of Ese-1 and SPRR2A expression (Fig. 4A).

To investigate whether Skn-1 and Ese-1 co-localize *in vivo*, we monitored the stratum-specific expression of Skn-1, Ese-1, and SPRR2A in sections of foreskin (Fig. 4B). Whereas Skn-1 (the 3'-UTR probe used detects all isoforms) was present in most suprabasal layers, Ese-1 expression was confined to the more differentiated layers. Most importantly, the distribution of both factors overlapped with the expression of SPRR2A. Because both octamer and Ets binding sites in the promoter are essential for SPRR2A expression (5), we questioned whether Skn-1 isoforms and Ese-1 had the potential to cooperate in SPRR2A transactivation.

*Ese-1 Selectively Cooperates with Skn-1a in the Transactivation of the SPRR2A Promoter*—HaCat cells were transfected with the SPRR2A-luciferase reporter construct (PSG350) together with increasing amounts of the different transcription factors either alone or in various combinations (Fig. 5). The total amount of transfected expression plasmid was kept constant by compensating with the empty vector. Skn-1a, Skn-1d1, and Ese-1 alone induced the SPRR2A promoter 3–4-fold at saturation. However, cotransfections of Skn-1a and Ese-1 activated the same promoter in a dose-dependent manner up to 8-fold, suggesting functional cooperativity between these two transcription factors (Fig. 5A). Such synergy was not found in combinations of Skn-1d1 with Ese-1 (Fig. 5B) nor with Skn-1a and Ets-2 (Fig. 5C), another Ets transcription factor previously implicated in keratinocyte terminal differentiation (30). Apparently, only Skn-1a can specifically cooperate with Ese-1 in the activation of the SPRR2A gene.

Fig. 5D shows the effect of Skn-1d1, Skn-1d2, or Ets-2 on the cooperative activation of the SPRR2A promoter by Skn-1a and Ese-1. Although neither Skn-1d2 nor Ets-2 was able to affect the cooperativity between Skn-1a and Ese-1, a clear drop in promoter activity was observed when Skn-1d1 was included. Apparently Skn-1d1 is able to compete with Skn-1a for promoter binding even in the presence of Ese-1, resulting in a complete abrogation of the synergistic effect. In the case of Ets-2 the situation is different; Ets-2 can at least partially down-modulate the activity of Skn-1a in the absence of Ese-1 (Fig. 5C) but not in its presence (Fig. 5D). Furthermore, it is shown that mutations in either Ets (PSG390) or the octamer binding site (PSG527) resulted in a complete inhibition of SPRR2A promoter activity, in agreement with our previously published results (5).

Taken together, our results show that although both Skn-1a and Skn-1d1 could transactivate the SPRR2A promoter, only Skn-1a was able to functionally cooperate with Ese-1, resulting in enhanced transactivation. Skn-1d1 was able to counteract this functional cooperativity.

#### DISCUSSION

In this study, we have identified and characterized three isoforms of the human Skn-1/Oct-11 gene and assessed their ability to regulate the human SPRR2A gene. The promoter of this gene has been well characterized in the past, and its activation during keratinocyte differentiation has been well documented. It encompasses an octamer binding site, that is recognized by Skn-1 and is essential for promoter activity together with three other transcriptional control elements bound by, respectively, the Ets, Irf, and Klf transcription factor families (5). Among these, only the Ets binding activity has previously been identified as the epithelial-specific Ets factor, Ese-1 (18). Our previous finding, that destruction of a single binding site results in a complete loss of promoter activity, has stressed the importance of signal integration and transcription factor cooperativity in the regulation of this gene (5). Here, we have

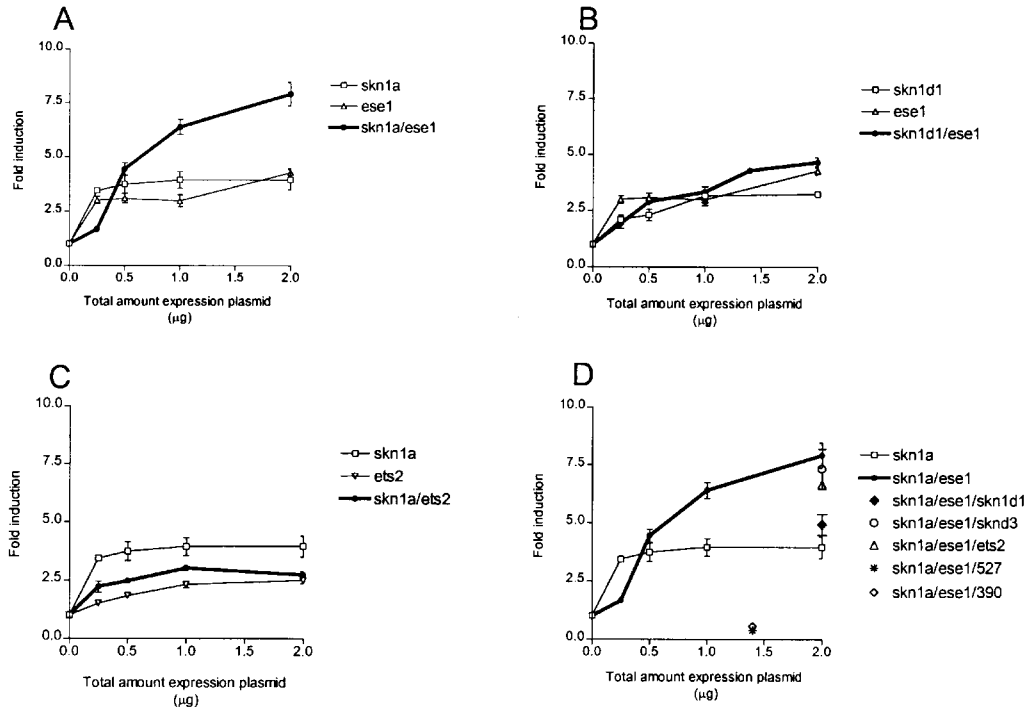


FIG. 5. Transcription factor cooperativity and antagonism during *SPRR2A* gene regulation; cotransfection of the *SPRR2A*-luciferase reporter construct (5 µg) with the indicated total amounts (µg) of expression plasmids for Skn-1a and/or Ese-1 (A), Skn-1d1 and/or Ese-1 (B), and Skn-1a and/or Ets-2 (C). When more than one transcription factor was transfected in the same mixture, the plasmids were always added in equal amounts. The total amount of transfected expression plasmid was kept constant at 2 µg by compensating with empty vector. D, cotransfection of the wild type (pSG350) or mutant (octamer site, pSG527; Ets site, pSG390) *SPRR2A* promoter constructs with Skn-1a/Ese-1 and the indicated transcription factor expression plasmids. Transfection and luciferase measurements were as described previously (28).

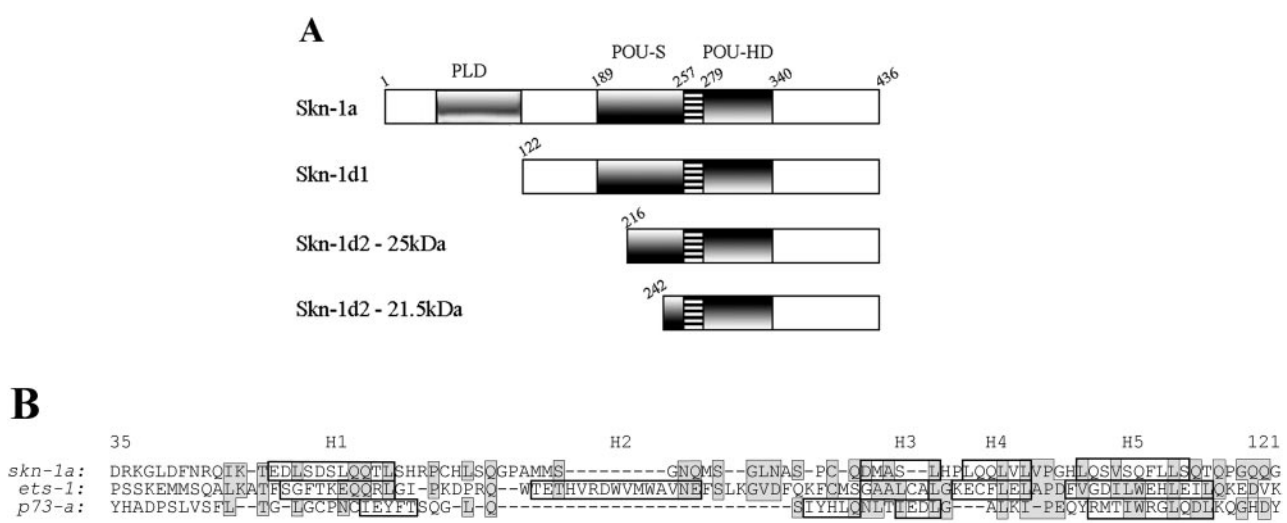


FIG. 6. A, schematic representation of the various Skn1 isoforms identified in this study. The POU-specific domain (*POU-S*), the POU-homeodomain (*POU-HD*), and the pointed-like domain (*PLD*) are represented. The amino acid numbering corresponds to the Skn-1a isoform. B, comparison of the N-terminal region (amino acids 35–121) of Skn-1a with the sterile  $\alpha$  motif/pointed domains of Ets-1 and p73- $\alpha$ . Identical and similar amino acid residues are highlighted in gray, and the predicted  $\alpha$ -helices in Skn-1a (3D-PSSM) and those in the resolved structures of Ets-1 (37) and p73 (38) are boxed. As a reference  $\alpha$ -helices in Ets-1 are numbered H1-H5.

used this well documented facet of *SPRR2A* regulation to analyze the regulatory abilities, including possible synergistic/antagonistic activities, of the three different Skn-1 isoforms that we have identified.

The human *Skn-1* gene produces three mRNA species, encoding proteins with various N termini (Fig. 6A). The two shorter mRNA variants are the result of internal promoter usage and initiate within introns 5 and 7. The observation that the three transcripts are differentially expressed in various skin samples and in cultured human keratinocytes (Fig. 4A)

indicates that the corresponding promoters function independently and are subjected to selective regulation. Although the Skn-1d1 and Skn-1d2 variants originate within introns, termination codons prevent the addition of specific N-terminal (“intron”-encoded) sequences to the proteins. Consequently, Skn-1d1 and Skn-1d2 can be viewed as N-terminal deletions of Skn-1a.

*Skn-1a*, which encodes the full-length protein, is identical to the mRNA previously described by others (29) and is highly homologous to its rat and mouse orthologues (13, 14). *Skn-1a* transcripts are expressed in all human skin samples analyzed

(Fig. 4A). The higher level of expression in epidermal samples (lanes 2–5) as compared with a total skin preparation (lane 1) correlates with the epidermal expression of this gene (Fig. 4B). Our analysis clearly shows that *SPRR2A* expression is not strictly linked with either *Skn-1A* or *Ese-1* expression, but it clearly correlated with the presence of both factors. For instance, low levels of *Ese-1* in uterus do not induce *SPRR2A* expression due to the absence of *Skn-1a* (Fig. 4A, lane 9). However, weak expression of *Skn-1a* can be compensated by the presence of *Ese-1* and results in efficient *SPRR2A* expression in cultured keratinocytes and cervix (lanes 7–8). These *in vivo* expression data are in line with our transient transfection experiments, which have established cooperativity of *Skn-1a* and *Ese-1* in *SPRR2A* promoter transactivation (Fig. 5) and corroborate the previously identified necessity for signal integration in the regulation of *SPRR2A* (5).

*Skn-1d1* did not have the ability to synergize with *Ese-1*. Because *Skn-1a* and *Skn-1d1* exhibited comparable DNA binding activities with the *SPRR2A* octamer site, the functional cooperativity between *Skn-1a* and *Ese-1* is likely to be mediated by a specific domain in *Skn-1a*, which is not present in *Skn-1d1*. Hence, the 122-amino acid N-terminal part of *Skn-1a*, which is absent in *Skn-1d1* (Fig. 6A), was screened for possible structural domains by using 3D-PSSM (31), a fold recognition program at Imperial Cancer Research Fund (London) ([www.sbg.bio.ic.ac.uk/3dpssm](http://www.sbg.bio.ic.ac.uk/3dpssm)). This search has revealed between amino acids 35 and 121 a region with significant similarity to the canonical sterile  $\alpha$  motif/pointed domain (Fig. 6B). This fold, which is found in many different proteins, including for instance the Ets and p53 transcription factor families, functions essentially as a protein-protein interaction interface (32, 33). It might seem tempting to speculate that the pointed-like domain (*PLD*) in *Skn-1a* interacts directly with the *Ese-1* pointed domain. However, such an interaction would not account for the highly specific cooperativity between *Skn-1a* and *Ese-1* that we have observed. *Ets-2* also contains a pointed domain; it has the ability to bind to the *SPRR2A* Ets site, but it does not synergize with *Skn-1a*. Consequently, it seems more likely that the *Skn-1a* pointed-like domain contacts a protein domain in *Ese-1*, which is specific for this factor.

The human *Skn-1d1* mRNA is conserved among rodents and primates; however, its coding potential has clearly changed during evolution. Although in primates the region encoded by intron 5 is not translated (due to in-frame termination codons), it encodes in rat and mouse an N-terminal 32-amino acid domain (Fig. 6A) that is responsible for the inhibitory activity of the *Skn-1i* isoform (13). *Skn-1d1* did also inhibit *SPRR2A* promoter activity in the presence of *Skn-1a* and *Ese-1* (Fig. 5D), although it had the ability to activate the same promoter when present on its own (Fig. 3). Furthermore, in our *in vivo* analysis, high *Skn-1d1* expression was inversely related to high *SPRR2A* expression. For instance in the epidermal samples of lanes 2, 3, and 5, where high expression of *Ese-1*, *Skn-1a*, and *Skn-1d1* is monitored, expression of *SPRR2A* is clearly lower than in foreskin (lane 6), where high expression of *Skn-1a* and *Ese-1* contrasts with low expression of *Skn-1d1*. Similarly, comparison of lanes 7 and 8 (cultured keratinocytes and cervix) reveals that similar levels of *Skn-1a* and *Ese-1* lead to lower expression of *SPRR2A* in the cultured cells (lane 7) due to the presence of *Skn-1d1*. The mechanisms by which h*Skn-1d1* and m*Skn-1i* inhibit gene expression differ, however, fundamentally. Whereas the m*Skn-1i* inhibitory domain acts in *cis* and inhibits DNA binding (13), the human counterpart has the same DNA binding affinity as *Skn-1a*. Due to this property it can compete with *Skn-1a* for octamer binding and interfere in this way with the cooperative activation of *SPRR2A* by *Skn-1a*

and *Ese-1*. The differential effect of *Skn-1d1* on *SPRR2A* expression in the presence or absence of *Skn-1a/Ese-1* is also interesting from a different point of view. It might actually shed light on several unexplained findings concerning the relative transactivation potential of *Skn-1a* and various truncated constructs, including an N-terminal deletion (29, 34). In these experiments the outcome depended greatly on the specific promoter that was tested. Although the N-terminal deletion of *Skn-1a* induced *K10* and *HPV1a* promoter activity, no discernible effect was observed on *HPV-18*, and *K14* was inhibited. Our results suggest that such variable outcomes can be expected and that they are likely to depend mainly on the specific transcription factor occupancy of the promoter that is analyzed. This is especially true for promoters subjected to strict combinatorial gene regulation, such as *SPRR2A* (5) and most likely also for the various promoters mentioned above. Consequently, to be able to fully appreciate the outcome of transcription factor truncation experiments, a reasonable knowledge of the regulatory configuration of the promoter that is analyzed is a prerequisite. A similar complexity has also recently been observed for the profilaggrin promoter and is discussed by the authors (7).

The human *Skn-1d2* isoform codes for two proteins lacking the first 27 or 53 amino acids of the POU-specific domain, whereas the POU homeodomain is left intact (Fig. 6A). It has previously been shown that both subdomains are required for DNA binding (35). This probably explains why *Skn-1d2* neither bound to the *SPRR2A* octamer site nor affected promoter activity. However, *Skn-1d2* was widely expressed, with transcripts detected in all skin samples analyzed. This feature suggests that *Skn-1d2* might have a physiological role, which does not depend on DNA binding. Indeed, POU domain proteins have been shown to regulate transcription also through protein-protein contacts, in a DNA binding-independent manner. These interactions are often mediated by the POU-specific, the POU-homeodomain, or both domains and target other transcription factors, co-regulators, basal factors, and chromatin components (for a recent review, see Ref. 36). For instance, down-regulation of keratin 14 is mediated by the POU domain of either *Skn-1a* or *Oct-6* and does not involve DNA binding to the *K14* promoter (34). In the case of *Oct-1*, several protein-protein interactions are mediated solely via the POU homeodomain (36). Consequently, it is possible that the *Skn-1d2*-encoded proteins, which still have intact POU homeodomains, have the ability to affect gene expression by similar protein-protein interactions. More experiments will be needed to unravel a possible regulatory function of the *Skn-1d2* isoforms.

Our work describes for the first time a functional interaction between the *Skn-1a* and *Ese-1* transcription factors. The differential cross-talk of *Skn-1a* and *Skn-1d1* with *Ese-1* highlights the complexity of combinatorial gene regulation during keratinocyte terminal differentiation. The strict dosage of *Skn-1* isoforms is likely to guarantee both the fine-tuning of the process of epidermal maturation and its adaptation to external and environmental hazards.

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**Distinct Functional Interactions of Human Skn-1 Isoforms with Ese-1 during  
Keratinocyte Terminal Differentiation**

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