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Oct-6: a Regulator of Keratinocyte Gene Expression in Stratified Squamous Epithelia

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POU domain proteins have been implicated as regulators of differentiation and development, particularly in early embryogenesis and in neural morphogenesis. Given that neural and epidermal lineages originate from a common precursor (ectodermal) cell, we explored the possibility that POU proteins are involved in epidermal differentiation. Using reverse transcription-PCR and degenerate oligonucleotides, we generated several POU domain cDNAs from cultured human epidermal mRNAs. One of these encoded a sequence identical to the rodent Tst-1/SCIP/Oct-6 POU domain. Subsequently, we isolated a cDNA encoding a 45.3-kDa protein with 98% sequence identity to rat Tst-1/SCIP and 94% identity to mouse Oct-6. This protein bound specifically to the canonical octamer motif, warranting its designation as human Oct-6. By RNase protection assays, by PCR, and by immunoblot analysis, Oct-6 was expressed in cultured epidermal keratinocytes. By in situ hybridization, Oct-6 mRNA was detected not only in epidermis but also a variety of other stratified squamous epithelia and with greater signals than testis, the tissue in which this POU protein was originally discovered. Moreover, Oct-6 exerted a marked and specific negative influence on expression of the K5 and K14 genes, abundantly expressed in most dividing stratified squamous epithelial cells and downregulated as cells commit to terminally differentiate. The repressive effect was complex, but it was not observed with Oct-1, nor was it seen with a truncated Oct-6 missing the POU domain. Taken together, our studies suggest that Oct-6 may play an important role in controlling gene expression in stratified squamous epithelia, including epidermis.

The temporal commitment of a cell to a particular program of differentiation is determined by a procession of regulatory molecules expressed in overlapping developmental and spatial patterns and culminating in the activation of a group of cell-type-specific structural genes. One group of transcription factors which can render global effects on programs of gene expression are the POU domain proteins (for reviews, see references 36, 37, 39, and 55). Initially discovered as a nematode developmental control gene, a pituitary-specific transcription factor, and a B-lymphocyte regulatory factor, these proteins constitute a growing family of transcription factors whose differential expression often coincides with critical stages of cell determination and tissue development.

The diagnostic feature of POU proteins is a 147- to 156-amino-acid POU domain, consisting of a 75- to 82-residue POU-specific segment and a 60- to 62-residue POU homeodomain separated by a short linker segment of more variable size and sequence. Analyses of several POU domain proteins have revealed underlying roles for the POU-specific domain and POU homeodomain in high-affinity, site-specific DNA sequence recognition and in protein-protein interactions. These proteins have been subdivided into five groups (POU-I to POU-V), based on slight variations in POU domain sequences that may confer subtle differences in DNA binding specificities (for reviews, see references 36, 37, and 55). Outside the POU domain, these proteins are diverse in sequence and size, even within members of a single class. Since these hypervariable regions also harbor the transcriptional control domains of the POU proteins, this diversity contributes to the differences in regulatory effects portrayed by different POU members.

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Most if not all POU proteins bind as monomers to the canonical octamer DNA binding site, ATGCAAAT, and on this basis, these proteins have been renamed as octamer, or Oct, proteins (10, 39, 44). In addition, POU proteins can associate in solution with themselves or with other members of the POU family, and this dimerization can often be stabilized when multiple binding sites occur in tandem arrays within a promoter (23, 32, 50, 53). In one case, however, heterodimerization of POU proteins leads to inhibition of DNA binding and suppression of transcription (50). Interestingly, this repressor, referred to as I-POU, differs from an activator POU protein by only two amino acids in the homeodomain (51). The complexity of DNA binding among POU proteins is further deepened by the realization that (i) non-POU proteins can also form complexes with them and influence their binding to DNA (4, 19, 25, 40, 43, 56) and (ii) POU proteins can undergo transient phosphorylation, which can influence their activity (16, 34, 46). At least in part, the diversity in protein-protein interactions and/or posttranslational modifications may confer on different POU proteins the ability to recognize various DNA binding sites differentially and to act as both positive and negative regulators (see, for example, references 7, 34, 50, and 51).

On the basis of their elaborate expression patterns in brain development, POU (Oct) proteins have been implicated in cell determination and differentiation within the central and peripheral nervous systems. Given the common embryonic origin of neural and epidermal cells, we wondered whether POU proteins might also play a role in development and differentiation in the epidermis. To this end, we used reverse transcription-PCR (RT-PCR) to clone POU cDNAs from cultured human epidermal mRNAs. Easily identified in keratinocyte mRNA, but not present in fibroblast mRNA preparations, was an RT-PCR fragment encoding a POU domain identical to mouse Oct-6 (also known as SCIP and Tst-1), a member of the

class III POU domain proteins (15, 29, 45). In this report, we describe the isolation and complete cDNA sequence encoding the human homolog of Oct-6. Previously, Oct-6 had been known as a POU protein expressed in embryonal carcinoma cells, testis, myelinating glia, and differentiating oligodendrocytes (8, 15, 28–30, 45). We report here that Oct-6 is also expressed in a variety of stratified squamous epithelia, including the epidermis. Moreover, as judged by transfection assays, we show that Oct-6 has the ability to negatively and specifically regulate the promoters for the genes encoding keratins K5 and K14, the hallmarks of dividing keratinocytes, and genes which are downregulated upon commitment to terminally differentiate. The mechanism of action is complex, which may be reflective of the presence of Oct-6 in both dividing cells, in which K5 and K14 are expressed, and in differentiating cells, in which they are not.

MATERIALS AND METHODS

Cell culture. Primary newborn human epidermal keratinocytes and the human epidermal squamous cell carcinoma line SCC-13 were cultured as described previously (57). The human diploid fibroblast strain WI-38, the human liver hepatocyte line HepG2, the human adenocarcinoma line HeLa, and the monkey kidney epithelial line COS were obtained from the American Type Culture Collection (Rockville, Md.) and cultured as described previously (22).

RT-PCR generation of POU domain sequences. RNAs were isolated as described by Chomczynski and Sacchi (6), except for testis RNA, which was purchased from Clontech Laboratories, Palo Alto, Calif.) Poly(A)$^+$ RNAs were purified by oligo(dT)-cellulose chromatography. To synthesize single-stranded cDNA, 2.5 μg of total RNA and 50 ng of random hexamer primers (Pharmacia, Piscataway, N.J.) in 14 μl of distilled H_2O were first heated to 70°C for 10 min and quickly chilled on ice. The following components were then added: 2 μl of 10× synthesis buffer (200 mM Tris-HCl [pH 8.4], 500 mM KCl, 25 mM $MgCl_2$, 1 mg of bovine serum albumin per ml), 1 μl of deoxynucleoside triphosphate mix (10 mM each dATP, dCTP, dGTP, and dTTP), 2 μl of 0.1M dithiothreitol, and 1 μl (200 U) of SuperScript reverse transcriptase (GIBCO BRL, Gaithersburg, Md.). After incubation at 42°C for 50 min, reactions were heated to 90°C for 5 min. Upon cooling to 37°C, 2 U of RNase H (GIBCO BRL) was added, and after 20 min, synthesized cDNAs were extracted, precipitated, and resuspended in 50 μl of 10 mM Tris (pH 8.1)–1 mM EDTA.

One microliter of each cDNA was used in PCR amplifications under conditions specified by Perkin-Elmer Cetus (Norwalk, Conn.). The following oligonucleotide primers were used: R, 5′-CTTGAATTCG(A/G)TTGCAGAACCAGACN CG-3′ (8-fold degenerate); R2, 5′-CTTGAATTCGGTTGC AGAACCAGACGCG-3′; L, 5′-CTTGAATTCGNCGNATC AA(A/G)CTNGG-3′ (128-fold degenerate); and L2, 5′-CT TGAATTCGGCGCATCAAGCTGGG-3′. Primers contained *Eco*RI sites at their ends to facilitate subsequent cloning. After 35 cycles at 94°C (1 min), 60°C (1 min), and 72°C (3 min), amplified DNAs were extracted and analyzed by electrophoresis through 3% NuSieve–1% SeaKem (FMC Corp., Rockland, Maine) agarose gels.

Bands of the expected sizes for POU domain cDNAs (380 to 430 bp) were excised from the gel. DNAs were electroeluted, purified, digested with *Eco*RI, and subcloned into the *Eco*RI site of pKS+ (Stratagene, La Jolla, Calif.). Cloned POU domain cDNAs were subjected to DNA sequencing with T3 and T7 primers as described by Sanger et al. (38).

Cloning of human Oct-6 cDNA. Phage from a λgt11 human epidermal cDNA library (Clontech) were screened (10 dishes at 5×10^4 plaques per 100-mm-diameter dish) in duplicate with (i) a PCR-generated 390-bp [^{32}P]dCTP-labeled probe sharing 100% sequence identity with the murine Oct-6 POU domain (specific activity, 8×10^8 cpm/μg) and (ii) a PCR-generated 420-bp [^{32}P]dCTP-labeled probe sharing 100% sequence identity with the human Oct-1 POU domain (specific activity, 6×10^8 cpm/μg). Probes were synthesized by using random primers and a random-priming kit (Stratagene). After hybridization at 42°C for 18 h, filters were washed in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) for 30 min each time at 37, 60, and finally 65°C. Plaques hybridizing with the Oct-6 probe, but not the Oct-1 probe, were further purified. Two independent clones were isolated, and the *Eco*RI inserts (800 and 2,200 bp) were subcloned into pKS+. Inserts were sequenced in both orientations by the method of Sanger et al. (38). Sequences were analyzed by using DNA Star sequencing programs (Beckman Research Laboratories, Pasadena, Calif.).

Construction of recombinant clones. Clones described previously are pK14CAT(−2300) (22), pK14CAT(DFPF) [same as pK14CAT(−1700/−270) (21)], pBLCAT2 (24), pBLDIST CAT (21), and pK5CAT(−6000) (5, 23a). p5XAP1tk-CAT, containing five synthetic copies of the human collagenase promoter's AP1 sequence inserted 5′ from the herpes simplex virus thymidine kinase (*tk*) promoter, was a gift of Michael Karin (University of California, San Diego). pACT-CAT, containing 4,300 bp of 5′ promoter sequence from the human β-actin gene, was a gift of Elwood Linney (Duke University Medical Center, Durham, N.C.). Plasmid pK14βgal(−2100) was constructed by inserting a 2,100-bp *Ava*I-*Ava*I fragment, extending from the transcription initiation site of the K14 gene to 2,100 bp 5′ of this site, into the *Xho*I site of pNASSβ (Clontech), using blunt-end ligation. Plasmid pK14CAT (−270), containing sequences −270 to +1 of the K14 gene, was made by endonuclease removal of sequences −2300 to −270 from pK14CAT(−2300) and religation. Plasmid pK14CAT(BCD) was prepared by inserting a 452-bp fragment, encompassing nucleotides (nt) −2098 to −1646 of the K14 gene, into the blunted *Acc*I site of pK14CAT(−270). Plasmid pK14CAT(CD) was prepared by inserting a 331-bp fragment containing nt −1977 to −1646 into pK14CAT(−270). Plasmid pK14CAT(D) was prepared by inserting a 160-bp fragment (nt −1814 to −1646 of the K14 promoter) into pK14CAT(−270).

Plasmid pKSOct1, containing human Oct-1 cDNA, was constructed by inserting a 2.5-kb *Kpn*I-*Hin*dIII fragment from pBSOct1 (44) into the *Kpn*I-*Hin*dIII site of pKS+. To construct plasmids pSGhOct6 and pSGhOct1, the full-length cloned Oct-6 and Oct-1 cDNAs, respectively, were subcloned into the dual prokaryotic-eukaryotic expression vector pSG5, containing a T7 bacterial promoter, followed by the major early promoter and enhancer from simian virus 40 followed by intron 1 from the rabbit β-globin gene, followed by a polylinker (13). pSGhOct6ΔC was made by linearizing pSGhOct6 with *Xho*I, filling in the ends with Klenow enzyme, and resealing, thereby generating a frameshifted version of Oct-6, missing most of the POU homeodomain (29).

Transfection of COS cells, preparation of nuclear extracts, and mobility shift assays. COS cells (simian virus 40 large T antigen positive) were seeded at 5.5×10^5 cells per plate. After 18 h, cells were transfected (12) with 10 μg of carrier pKS(+) DNA and 10 μg of either pSG5 or pSGhOct6. Cells were harvested 48 to 64 h after transfection, and nuclear extracts were prepared (22). Phenylmethylsulfonyl fluoride was

added to 2 mM in all solutions. Protein concentration were measured by the method of Bradford (3).

For nuclear extract binding studies, the following double-stranded DNA probes were generated: Oct-1 (5′-TGTCGAA TGCAAATCACTAGAA-3′), containing an immunoglobulin promoter canonical octamer motif, and AP2 (5′-GATCGAA CTGACCGCCCGCGGCCCGT-3′), containing the AP2 binding site of the simian virus 40 promoter (Promega Biotec, Madison, Wis.). Electrophoretic mobility shift assays (EMSAs) were performed as described elsewhere (21) with either 1 μl of programmed rabbit reticulocyte lysate or nuclear extracts (COS [1 μg] and SCC-13 [5 μg]). Unlabeled competitor oligonucleotides were used at a 50-fold molar excess over labeled probe.

Transient transfections and CAT/β-Gal assays. SCC-13 cells were transfected with 6×10^{-12} mol of chloramphenicol acetyltransferase (CAT) reporter plasmids, 10^{-12} mol of plasmid pCH110βgal (Pharmacia), and 4×10^{-12} mol of either pSG5, pSGhOct6, pSGhOct1, or pSGhOct6ΔC (21). The total amount of DNA was adjusted to 50 μg with pKS+. Soluble proteins were extracted, quantitated, and assayed for picograms of CAT protein per microgram of cell protein via a colorimetric sandwich enzyme-linked immunosorbent assay (ELISA) method (Boehringer Mannheim). β-Galactosidase (β-Gal) expression from cotransfected pCH110 enabled monitoring of transfection efficiency and normalization of CAT values. Extracts were assayed in duplicate, within the linear range of standard curves.

In situ hybridizations. Tissues were fixed in 4% paraformaldehyde for 6 h at room temperature, embedded in paraffin, and sectioned (5 μm). Sections were subjected to in situ hybridization as described previously (18). [^{35}S]UTP-labeled cRNAs were generated from (i) SP6 polymerase and *Hin*dIII-linearized p3SP, containing 1,080 bp of human K14 coding sequence (18); (ii) SP6 polymerase and *Bam*HI-linearized phK10-3′, containing 391 bp of 3′ untranslated region of human K10 mRNA (42); (iii) T7 polymerase and *Xho*I-linearized pM14-3′nc, a derivative of pKS+ containing 249 bp of 3′ sequence of mouse K14 mRNA (17, 52); (iv) T7 polymerase and *Bst*BI-linearized pKSOct6, producing a 673-nt (residues 1504 to 2170), 3′ untranslated cRNA of human Oct-6; (v) T7 polymerase and *Hin*dIII-linearized phOct6-5′, a pGEM3Z derivative of pKSOct6 containing an *Sph*I-*Pvu*II fragment encompassing 469 bp of coding sequence (residues 152 to 624) 5′ to the POU domain; or (vi) T7 polymerase and *Hin*dIII-linearized pmuLor-1, containing a 2.9-kb *Eco*RI fragment of mouse loricrin cDNA (15a).

Nucleotide sequence accession number. The sequence reported has been assigned GenBank accession number L26494.

RESULTS

Human Oct-6 mRNA: a familiar mRNA in an unfamiliar place. To ascertain whether epidermal keratinocytes express POU domain mRNAs distinct from the ubiquitous Oct-1, we used degenerate oligonucleotide primers and RT-PCR to amplify highly conserved POU domain sequences. One ~400-bp cDNA was specifically amplified in human keratinocyte mRNA but not fibroblast mRNA (Fig. 1). Its predicted amino acid sequence was 100% identical to the POU domains of (i) rat Tst-1, i.e., SCIP, discovered in testes (15) and sciatic nerve (30), respectively, and (ii) murine SCIP/Oct-6, the mouse Tst-1/SCIP homolog isolated from embryonal cells (28, 45). Indeed, RT-PCR conducted on human testis RNA produced the same-size POU band, consistent with the notion that this might be the human homolog of Tst-1/SCIP/Oct-6.

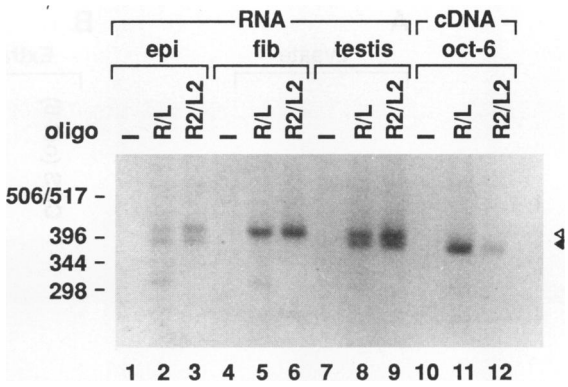

FIG. 1. RT-PCR generation of cDNAs with sequence homologies to POU domains. Human RNAs from epidermal cells (epi), WI-38 fibroblasts (fib), and testis were subjected to RT-PCR analysis using oligonucleotide primers corresponding to the highly conserved regions of mammalian POU domains. One set of specific (R2/L2) and one set of degenerate (R/L) oligomers were used (see Materials and Methods). Fragments were resolved by gel electrophoresis and then visualized by ethidium bromide staining. Migration of DNA markers is indicated at left in nucleotides. Each group of three lanes on the gel correspond to RT-PCR conducted (i) in the absence (–) of oligomers, (ii) in the presence of the R/L set, and (iii) in the presence of the R2/L2 set. The upper band (open arrowhead) corresponds to Oct-1, as suggested by use of a cloned human Oct-1 cDNA control in the RT-PCR and as verified by DNA sequencing (data not shown). The lower band (solid arrowhead) corresponds to Oct-6, as suggested by use of the subsequently cloned human Oct-6 cDNA as a control (lanes 10 to 12), and as verified by DNA sequencing and comparison with mouse and rat Oct-6 sequences (data not shown).

When this POU domain fragment was used to screen a human keratinocyte cDNA library, one isolate contained an ~2,200-bp cDNA insert which was then subcloned into the bacterial plasmid pKS+ and the mammalian expression vector pSG5 to produce pKSOct6 and pSGhOct6, respectively. When pKSOct6 was subjected to transcription and translation in the presence of [^{35}S]methionine (see Materials and Methods), a protein which migrated on SDS-polyacrylamide gels as 46 kDa was produced (Fig. 2A). Produced both by pSGhOct6-transfected COS cells and by reticulocyte lysates, this protein cross-reacted with an anti-rat SCIP antiserum (Fig. 2B). Moreover, an anti-SCIP-reactive protein of this size was also detected in nuclear extracts from epidermal cells but was not readily discernible in hepatocytes (HepG2), fibroblasts (WI-38), or simple epithelial cells (HeLa) (Fig. 2B). Collectively, these data demonstrated that a SCIP-related mRNA and its encoded protein are both specifically expressed in epidermal cells.

Sequence analyses confirmed that we have cloned the human homolog of Tst-1/SCIP/Oct-6 (Fig. 3). The cloned fragment is 2,170 bp in length and contains an open reading frame of 1,344 bp, with 41 and 785 nt, respectively, of putative 5′ and 3′ untranslated sequence. The cDNA encodes a protein of 448 amino acids (predicted molecular mass of 45.3 kDa) which shares 94% sequence identity with mouse Oct-6 and 98% identity with rat Tst-1 (SCIP). The amino-terminal portion possesses the alanine- and glycine-rich sequences previously shown to be important for the transactivation properties of mouse Oct-6 (27).

Recently, a different POU protein encoded by a cloned human fetal brain was claimed to be the human homolog of Oct-6 (48). The 5′ end of this other cDNA sequence is distinct from our cDNA and from rodent Oct-6, and it has an open

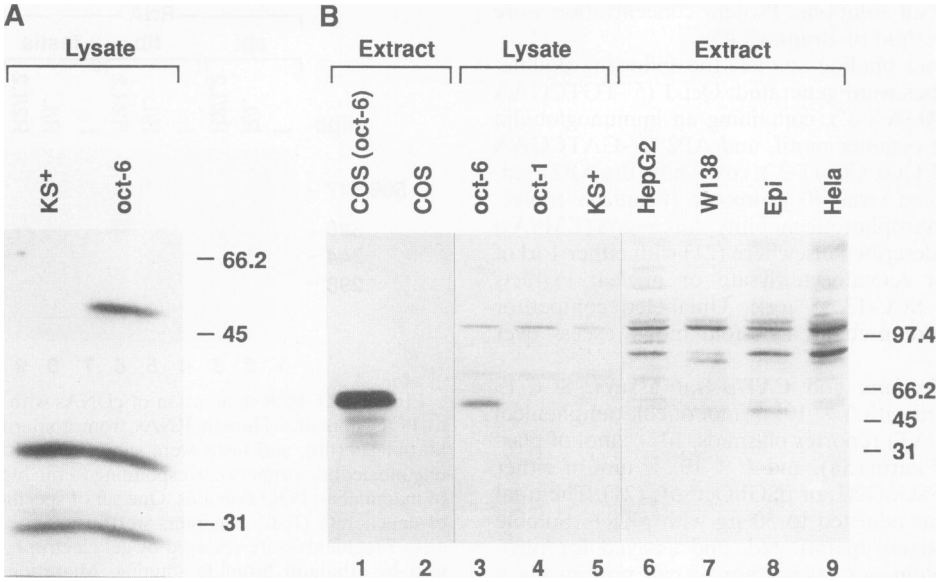

FIG. 2. The cloned human POU cDNA encodes a protein related to rat SCIP and present in epidermal keratinocytes. (A) In vitro synthesis of radiolabeled proteins was performed by using a transcription-translation rabbit reticulocyte kit (TNT T3-T7; Promega) supplemented with 4 μCi of [^{35}S]methionine (specific activity, 1,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) and 1 μg of either pSG5 (KS+), pSGhOct6 (oct-6), pSGhOct6ΔC (not shown), or pSGhOct1 (not shown). Translated proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE). Migration of protein standards is given at right (in kilodaltons). (B) Transcription-translation lysate assays with pKSOct6 (oct-6), pKSOct1 (oct-1) or pKS+ (KS+) were prepared as for panel A but with unlabeled, not labeled, methionine. In addition, we prepared soluble extracts from COS cells transfected with pSG5 (COS) or pSGhOct6 [COS(oct6)] and nuclear extracts from HepG2 hepatocytes, WI38 fibroblasts, primary epidermal cells (epi), or HeLa epithelial cells. Lysates (5 μl), COS extracts (5 μg), and nuclear extracts (25 μg) were resolved by SDS-PAGE and subjected to immunoblot analysis using a rabbit polyclonal anti-SCIP antiserum (1:10 dilution).

reading frame that predicts a protein 50 amino acids shorter than our cDNA. We later show that our protein not only can bind to a canonical octamer element but also can function as a transcriptional regulator. Thus, we will refer to our protein hereafter as human Oct-6. The possible significance, if any, of the putative shorter Oct-6 must await characterization of the human Oct-6 gene and characterization of the other protein. However, it is notable that the rat Oct-6 gene is devoid of introns (20), and there is no canonical splice junction at the site where the two human cDNAs diverge in sequence. Hence, although differential splicing of some POU genes has been reported (47), differential splicing would be unlikely if the human Oct-6 gene structure conforms to that of the published rat Oct-6 gene.

Human Oct-6: a bona fide octamer-binding protein. The Oct protein classification is based on ability to bind to the canonical octamer motif. To test whether our protein warranted such a designation, we performed EMSAs with a radiolabeled double-stranded oligomer containing the canonical octamer motif, ATGCAAAT, combined with transcription and translation mixtures programmed with pKSOct1 (control) or pKSOct6 (test). Both the pKSOct1-primed and the pKSOct6-primed lysates synthesized proteins which bound to the octamer oligomer (Fig. 4A). The mobility shift was markedly smaller for human Oct-6 than for Oct-1, consistent with prior gel shift results on rodent Oct-6 proteins (20, 28, 45).

As judged by EMSA, extracts from untransfected or mock-transfected COS cells possess endogenous POU proteins (Fig. 4A). The major species appears to be Oct-1, as judged by the similar mobility of this band and the pKSOct1-programmed lysate band. Interestingly, when octamer probe was incubated with nuclear extract from pSGhOct6-transfected COS cells, the endogenous Oct-1 band diminished (visible on longer exposures) and a strong exogenous Oct-6 band appeared. The absence of free probe at the gel front of this lane was suggestive that the excessive Oct-6 present in this extract competed effectively with endogenous Oct-1 for a limiting amount of probe.

When octamer oligomer was incubated with nuclear extract from primary human epidermal keratinocytes, two bands were generated (Fig. 4A, epi). The strongest band corresponded to the Oct-1 band, and the weaker one corresponded to the Oct-6 band (arrowheads). The anti-SCIP antibody did not function in supershift assays, and consequently, we were unable to confirm the identity of the Oct-6 band. However, both bands were competed for effectively with cold octamer element (epi + oct). These findings are consistent with our RT-PCR data, our ability to clone Oct-6 from an epidermal cDNA library, and our immunoblot analyses indicating that a 46-kDa anti-SCIP-reactive Oct-6 protein is present in epidermal nuclear extracts.

Finally, to verify specific binding between our recombinant POU proteins and the octamer probe, unlabeled oligomer (oct) was used at a 50-fold excess in competition EMSAs (Fig. 4B). Unlabeled octamer competed effectively with radiolabeled probe for the binding of both Oct-1 and Oct-6 proteins. In contrast, competition was not appreciable with an oligomer containing a binding site for AP2, a transcription factor involved in the regulation of many keratinocyte genes (21, 41). Taken together with the EMSAs in Fig. 4A, these studies demonstrate convincingly that the human homolog of Tst-1/SCIP/Oct-6 is indeed an octamer-binding protein and is deserving of the name Oct-6.

Quantitation of Oct-6 RNA in epidermal keratinocytes. To quantitate the levels of Oct-6 RNA expression in cultured keratinocytes, we conducted RNase protection assays (Fig. 5). Incubation of a synthetic Oct-6 RNA with a 520-nt radiola-

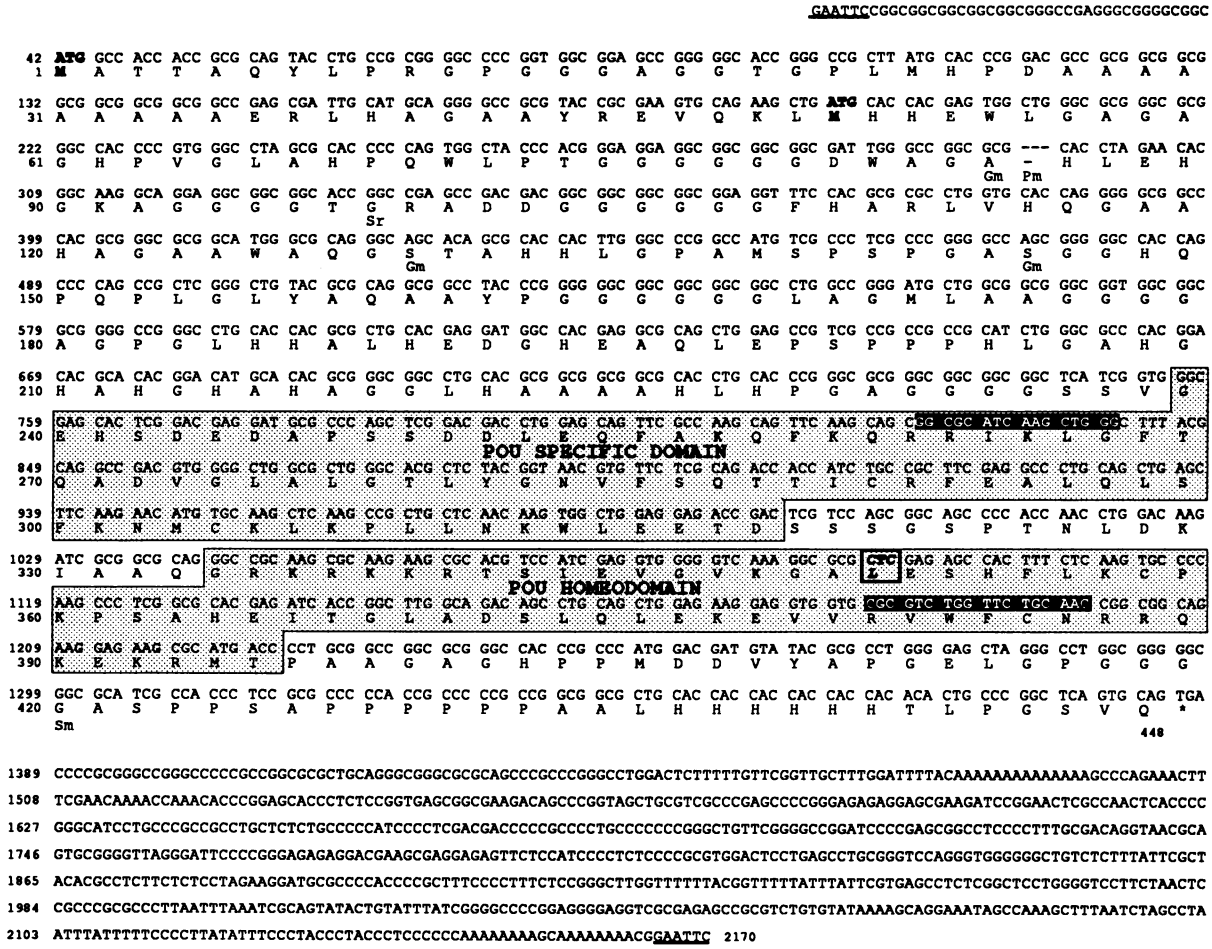

FIG. 3. Nucleotide sequence and predicted amino acid sequence of human Oct-6 cDNA. The predicted translation start codon (ATG) is shown as amino acid 1, in agreement with the rodent translation initiation site. The putative translation start codon suggested by Tobler et al. (48) is also highlighted; this is an internal methionine both in the rodent Oct-6 sequence and in our human sequence. The POU-specific domain and the POU homeodomains are boxed in grey, and the sequences recognized by our degenerate primers are boxed in black. Amino acids that differ in the mouse or rat sequences are noted as Xm or Xr, respectively. Note that the amino end of the protein contains a stretch of 9 alanines in the mouse and human sequences and 11 in the rat sequence; also, there is no perfect consensus polyadenylation signal, nor did our cDNA clone end in a stretch of poly(A). The boxed leucine at position 351 is the last wild-type residue in Oct6ΔC, described later in the text. Artificial *Eco*RI sites used in subcloning are underlined.

beled antisense probe resulted in an RNA-RNA hybrid which when digested with RNase produced a 469-nt diagnostic fragment corresponding to the portion of the probe that was complementary to Oct-6 mRNA (Fig. 5; compare lane 2 [free probe] with lanes 4 to 7 [digested hybrids]). As little as 5 ng of Oct-6 RNA could be detected in this assay (lane 5). When the assay was repeated, this time with poly(A)-enriched keratinocyte RNA as opposed to synthetic Oct-6 RNA, a 469-nt fragment was again generated (lane 10). The relative level of Oct-6 RNA was calculated to be ~0.03% of the keratinocyte RNA. Thus, even though present in low abundance, Oct-6 mRNA is appreciable in epidermal cells and not merely a minor component amplifiable by PCR.

Human Oct-6 cRNAs hybridize broadly to many stratified squamous epithelia. Since Oct-6 had previously been implicated only in the differentiation of testes, myelinating Schwann cells, oligodendrocytes, and embryonic carcinoma cells, we were surprised to detect Oct-6 mRNA in keratinocytes. We examined more closely the expression of Oct-6 in skin by conducting in situ hybridizations. To control for basal-specific expression, we used a keratin 14 cRNA probe. K14 along with its partner K5 constitute the major structural proteins of basal keratinocytes (9, 31). To control for suprabasal-specific expression, we used K10 cRNA as an early marker of terminal differentiation (9, 35). Finally, to verify that our hybridization was specific for Oct-6, and not an artifact of either nonspecific hybridization with a portion of the probe or more general hybridization with related POU RNAs, we used two different Oct-6 probes: one 5′ from the POU domain and the other to 3′ untranslated sequence.

Figure 6 illustrates the results of the in situ hybridizations. As expected from previous studies (reference 18 and references therein), the human K14 cRNA hybridized specifically and abundantly to the basal layer of epidermis (Fig. 6A [bright field] and B [dark field]). In contrast, the human K10 cRNA hybridized to the suprabasal layers (Fig. 6C and D), similar to that seen previously with a K1-specific cRNA (35). In contrast to K14 and K10 cRNAs, both the 5′ and the 3′ Oct-6 cRNAs hybridized throughout the living layers of the epidermis (Fig. 6E and F and Fig. 6G, respectively).

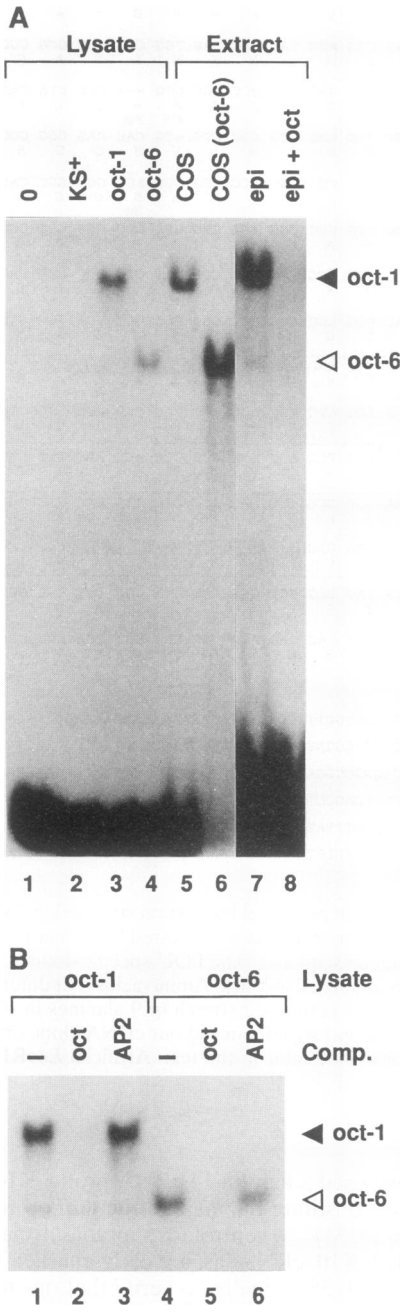

FIG. 4. Binding of human Oct-6 to the canonical octamer motif. (A) Radiolabeled probe containing an octamer motif was used in EMSAs with unlabeled protein generated from (i) transcription-translation lysate reactions containing no added plasmid (0; lane 1), pKS+ (KS+; lane 2), pKSOct1 (oct-1; lane 3), and pKSOct6 (oct-6; lane 4; (ii) extracts (5 μg) of untransfected COS cells (lane 5) or COS cells transfected with pSGhOct6 (lane 6); and (iii) nuclear extracts (20 μg) of primary epidermal keratinocytes (epi) without (lane 7) or with (lane 8) unlabeled octamer (oct) competitor. DNA-protein complexes are identified at right. (B) EMSAs on transcription-translation reactions with in vitro-generated Oct-1 (lanes 1 to 3) or Oct-6 (lanes 4 to 6) were repeated, this time using unlabeled octamer (oct) or AP2 competitor (Comp.) oligomers in 50-fold excess.

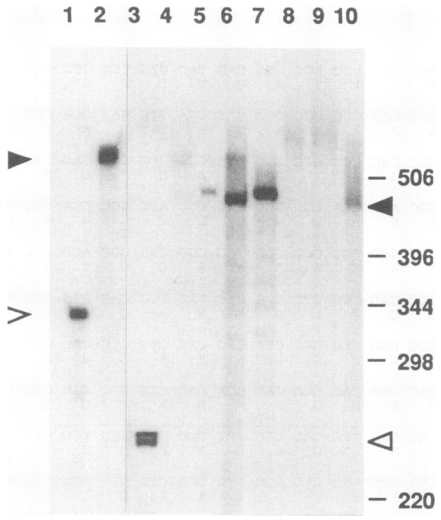

FIG. 5. RNase protection assay to quantitate Oct-6 mRNA levels in cultured keratinocytes. RNase protection assays were carried out with an Ambion RPA II kit (Ambion, Austin, Tex.). The test plasmid, phOct-6-5′, was a *Sph*I-*Pvu*II fragment of human Oct-6 cloned into the *Hin*dIII-*Sma*I site of pGem3Z. The plasmid, linearized with *Hin*dIII, was used with T7 RNA polymerase and radionucleotides to make radiolabeled antisense probe (520 nt), 469 nt of which were complementary to human Oct-6 mRNA sequence. The control plasmid, pTRI-β-actin-Human, was provided by Ambion, and a 325-nt radiolabeled antisense probe, ~250 nt of which were complementary to human actin mRNA, was prepared. mRNAs were either purchased from Clontech (human brain and testis RNA), isolated from cultured human keratinocytes, or synthesized in vitro, using T3 RNA polymerase and *Bam*HI-linearized pKSOct6. Lane 1, actin probe, prior to RNase; lane 2, Oct-6 probe prior to RNase; lane 3, actin probe after prehybridization with 20 μg of WI-38 fibroblast RNA, followed by RNase; lanes 4 to 7, Oct-6 probe after protection with 1, 5, 10, and 50 ng, respectively, of control Oct-6 RNA, followed by RNase; lanes 8 to 11, Oct-6 probe after RNase treatment of the probe protected with 20 μg of testis RNA (lane 8), 20 μg of brain RNA (lane 9), and 8 μg of poly(A)-enriched (~10-fold) keratinocyte RNA (lane 10). Exposure of lanes 3 to 10 was for 6 days (1-h exposures were used for lanes 1 and 2). DNA standards are given at right in base pairs. Arrowheads at left denote positions of undigested actin (open) and Oct-6 (solid) probes, while those at right denote positions of same probes after the RNase protection assay. Note that a 470-nt band corresponding to ~5 ng of Oct-6 RNA was seen in the keratinocyte mRNA protection assay, whereas under conditions used here, Oct-6 RNA levels in brain and testis were below the limits of detection.

Our Oct-6 probes were chosen for their lack of homology with other POU protein RNAs, an important feature given the recent report that mRNAs encoding Skn-1a and Skn-1i, POU proteins related or identical to Oct-11, are also expressed in epidermis (2). We found that a mouse Oct-11 cRNA showed basal rather than basal/spinous hybridization of mouse epidermis, thereby suggesting that the Oct-6 pattern of expression was distinct from that of other known skin POU RNAs (5a). Besides Oct-6 cDNA of other species, there were no sequences in GenBank that showed high homology with our Oct-6 probes. This said, because a low homology (11-nt stretch of identity) was found between the 5′ Oct-6 coding probe and loricrin, a high-abundance epidermal mRNA, we verified that cross-hybridization was not a problem. As seen in Fig. 6H, a loricrin cRNA probe gave granular layer labeling, clearly distinct from the pattern of Oct-6 cRNAs. Taken together, our data corroborate the specificity of our probes and suggest that Oct-6 mRNA or an Oct-6-related mRNA is expressed specif-

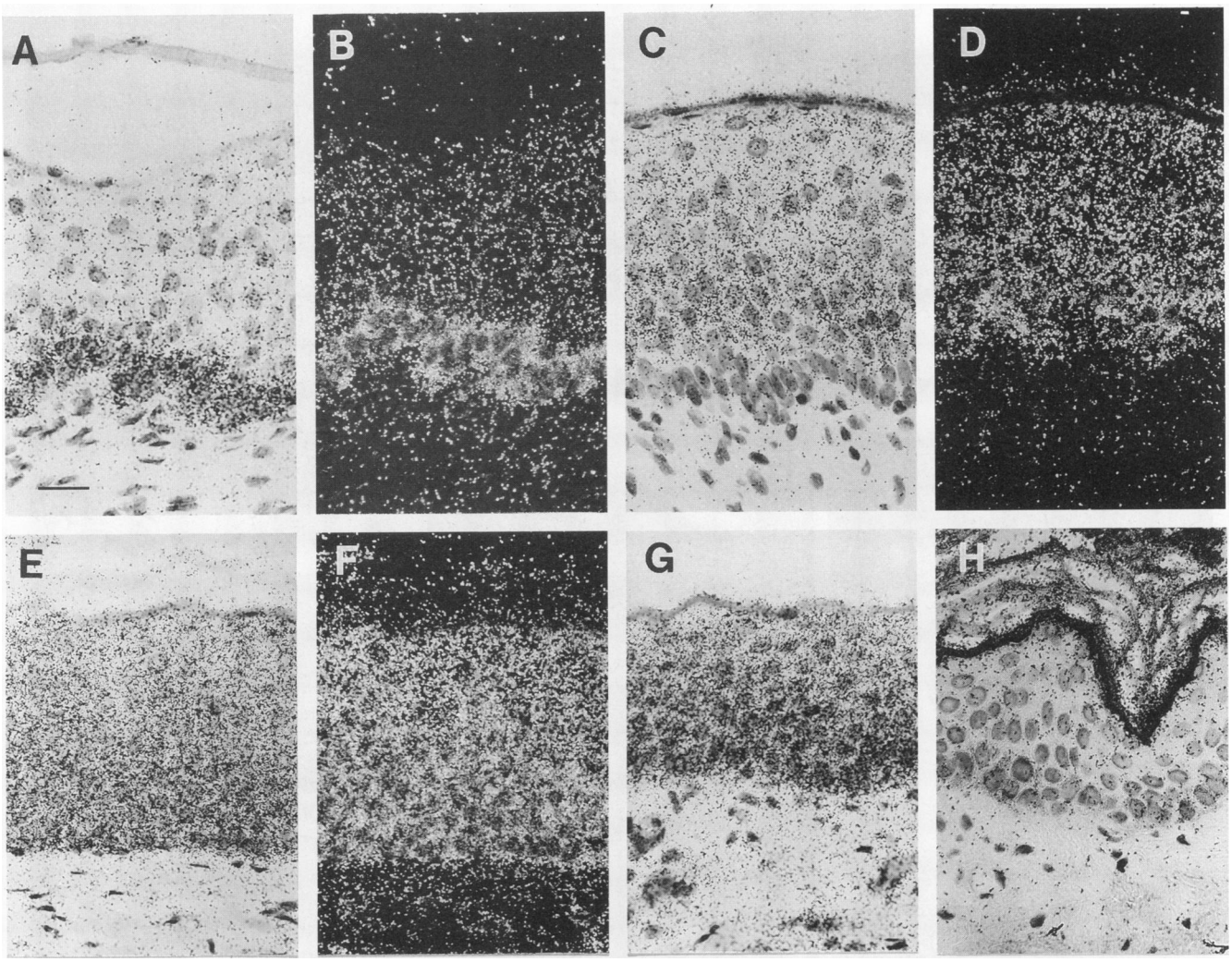

FIG. 6. In situ hybridizations of human K14, K10, and Oct-6 and mouse loricrin cRNAs to neonatal human epidermis. All cRNA probes were radiolabeled with [^{35}S]UTP and hybridized to sections (5 μm) of human neonatal foreskin as described in Materials and Methods. Tissue sections were counterstained lightly with hematoxylin and eosin and subjected to autoradiography for 1 to 4 weeks. Hybridizations were with cRNA probes to K14 (A [bright field] and B [dark field]), K10 (C [bright field] and D [dark field]), Oct-6 5′ probe (E [bright field] and F [dark field]), Oct-6 3′ probe (G), and loricrin probe (H). The bar represents 50 μm and applies to all frames.

ically in both basal and suprabasal layers of epidermis. Because of substantial cross-reactivity of anti-rat SCIP antiserum with other nuclear proteins (Fig. 2B), we could not assess whether the Oct-6 protein that we detected in nuclear extracts of keratinocytes (Fig. 2B and 4A) was expressed in an analogous pattern.

To ascertain whether the Oct-6 cRNA hybridization pattern might be a feature shared among stratified squamous epithelia, we took advantage of the high degree of sequence identity (93%) between the 5′-terminal half of human and mouse Oct-6 cRNAs and hybridized the human Oct-6 cRNA probe to sections of stratified squamous epithelia from various adult mouse tissues (Fig. 7). Hybridization with a mouse K14 cRNA control (17, 52) showed basal preferred labeling in all stratified squamous epithelia tested (shown are foot skin [Fig. 7A], ventral tongue [Fig. 7E], and dorsal tongue [Fig. 7G]). Oct-6 cRNA also hybridized to all stratified squamous epithelia, but in contrast to K14 cRNA, Oct-6 cRNA displayed hybridization throughout the metabolically active layers of the epithelium. Oct-6-positive hybridization included foot skin (Fig. 7B), forestomach (Fig. 7C [bright field] and D [dark field]), tongue (Fig. 7F [ventral surface] and H [dorsal surface]), vagina (Fig. 7I), and esophagus (Fig. 7J). Surprisingly, hybridization was not as strong in testis (Fig. 7K [bright field] and L [dark field]), the original source of the rat homolog (15). Collectively, these studies suggest that Oct-6 mRNA or an Oct-6-related mRNA is broadly expressed in stratified squamous epithelia. Within each epithelium, no specificity between basal and suprabasal expression was discernible.

Oct-6 is a repressor of K14 and K5 gene expression. K14 and K5 are the only keratins broadly expressed in all stratified squamous epithelia (31). In epidermis, they constitute up to 30% of total protein of basal cells (9), and much of this is due to abundant transcription of their genes (42). Previously, we isolated and characterized the human K5 and K14 genes and showed that their 5′ upstream sequences contain sufficient information to target expression to basal epidermal keratinocytes in transgenic mice (references 5 and 52 and references therein).

To ascertain whether Oct-6 might influence expression of

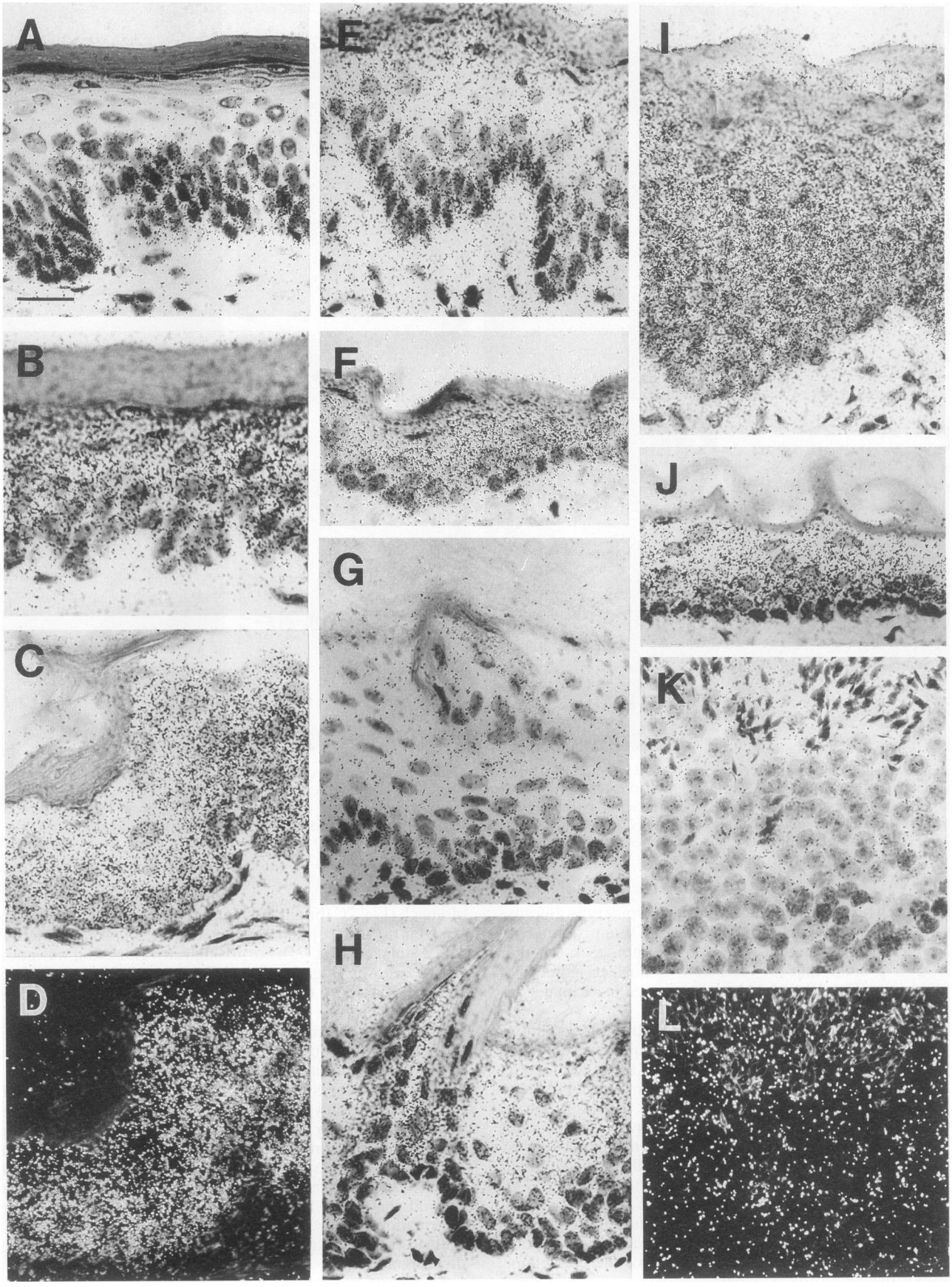
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FIG. 7. In situ hybridizations of human Oct-6 cRNAs to stratified squamous epithelia. Stratified squamous epithelial tissues from the mouse were sectioned, counterstained lightly with hematoxylin and eosin, and subjected to autoradiography for 3 weeks. Hybridizations and probes used are foot, K14 (A); foot, Oct-6, (B); forestomach, Oct-6 (C [bright field] and D [dark field]); ventral surface of the tongue, K14 (E); ventral tongue, Oct-6 (F); dorsal tongue, K14 (G); dorsal tongue, Oct6 (H); vagina, Oct-6 (I); esophagus, Oct-6 (J); and testis, Oct-6 (K [bright field] and L [dark field]). The bar represents 50 μm.

the K5 and K14 genes, we cotransfected cultured keratinocytes (SCC-13) with pSGhOct6 and hybrid keratin-CAT genes, containing various amounts of 5′ upstream sequence from the human K5 and K14 genes. A control plasmid (pCH110) was used to standardize for transfection efficiency, and all CAT values were normalized to this internal standard. Figure 8 illustrates the constructs that were tested, and Fig. 9 provides the results of these experiments, which were all performed in duplicate and at least two independent times.

CAT expression of pK14CAT(−2300), containing 2,300 bp of the human K14 promoter (22), was arbitrarily set at 100%. The empty expression vector, pSG5 (−Oct-6 in Fig. 9), had no effect on this level, whereas pSGhOct6 (+Oct-6 in Fig. 9) markedly (more than threefold) suppressed expression. This effect could not be attributed to the CAT sequences, since Oct-6 also repressed expression of pK14βgal(−2100) (in this case, pACT-CAT was used as an internal standard). Interestingly, pK5CAT(−6000), containing 6,000 bp of human K5 promoter sequence (5), was also repressed by pSGhOct6, and repression was even greater (about fivefold) than that seen with the K14 promoter. In contrast, no effect of Oct-6 was seen on expression of pACTCAT, containing the human β-actin promoter, or on p5XAP1tk-CAT, containing five copies of a synthetic AP1 site inserted upstream from the herpesvirus *tk* promoter. The *tk* promoter alone (pBLCAT2) displayed only a minor repression by pSGhOct6. Thus, in the context of these studies, the repressive effects of pSGhOct6 seemed to be specific for the basal epidermal keratin genes and not merely reflective of general squelching.

To determine whether these repressive effects were unique to Oct-6 or whether they might be a general phenomenon of POU proteins, we repeated the pK14CAT(−2300) assays, this time with pSGhOct1, i.e., the Oct-1 analog of pSGhOct6 (+Oct-1 in Fig. 9). In this case, only a slight drop (~25 to 30%) in expression was observed. It is interesting that Oct-6 seems to be the major repressor of basal keratin gene expression, since Oct-1 is far more abundant in basal keratinocytes, where the K5 and K14 promoters are active.

Intriguingly, the negative effects of Oct-6 were completely obliterated with a mutant, Oct-6ΔC, missing most of the POU homeodomain necessary for DNA binding. Using the transcription-translation reticulocyte lysate assay, the frameshift mutant was expressed in vitro at comparable levels to the wild-type Oct-6 (not shown), making it unlikely that differences in mRNA translation or protein stability accounted for the differential behaviors of Oct-6 and Oct-6ΔC. Moreover, previous studies with a similarly designed rat SCIP mutant demonstrated that unlike wild-type SCIP, the frameshift mutant was unable to repress a myelin gene promoter in cultured Schwann cells (29). This said, we cannot exclude the possibility that the truncation altered protein binding domains in addition to the POU DNA binding domain.

Previously, we partially characterized 5′ upstream sequences regulating expression of the human K14 gene (21, 22). We showed that a proximal and a distal element were sufficient for expression in keratinocytes in vitro and in transgenic mice in vivo (22). To determine whether pSGhOct6 might negatively affect these elements, we tested plasmid pK14CAT(DFPF),

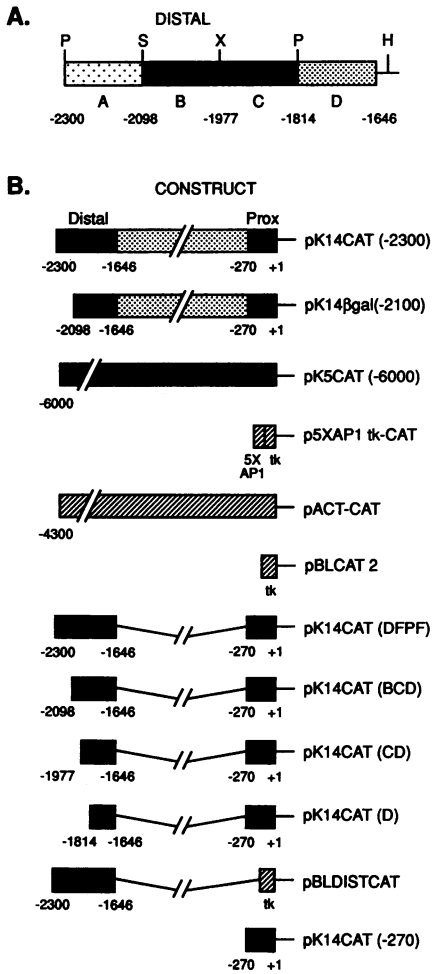

FIG. 8. Genetic maps of CAT/β-Gal constructs used in keratin promoter assays. (A) Map of the distal element (nt −2300 to −1646, where +1 is the transcription initiation site) of the K14 promoter (22). The element is subdivided into four segments (A to D). Restriction endonuclease sites used for subdividing: P, *Pst*I; S, *Sma*I; X, *Xho*I; H, *Hin*dIII. (B) Constructions of clones are described in Materials and Methods. Black bars, 5′ upstream sequences of the human K14 gene (22). The proximal promoter extends from +1 to a *Bsu* 36 site at −276 (transcription initiation site = +1). The distal promoter extends from a *Bst*XI site to a *Hin*dIII site, spanning sequences −1646 to −2300. Dotted bars, K14 gene sequences −270 to −1646, which were dispensable in CAT assays performed in transiently transfected cultured keratinocytes (22) and were omitted wherever indicated by a thin bar instead of dotted bar. Hatched bars, the promoter sequences (−105 to +51) of the herpesvirus *tk* gene with or without the 5× AP1 site sequences as indicated. Hatched bars also denote the 4,300 bp of the human β-actin promoter in pACT-CAT. The upstream sequence of the human K5 gene, extending 6,000 bp 5′ from the translation initiation site, is in grey. Note that all promoters are linked to the CAT reporter, except one, which was linked to the β-Gal reporter. Plasmid names are given at the right.

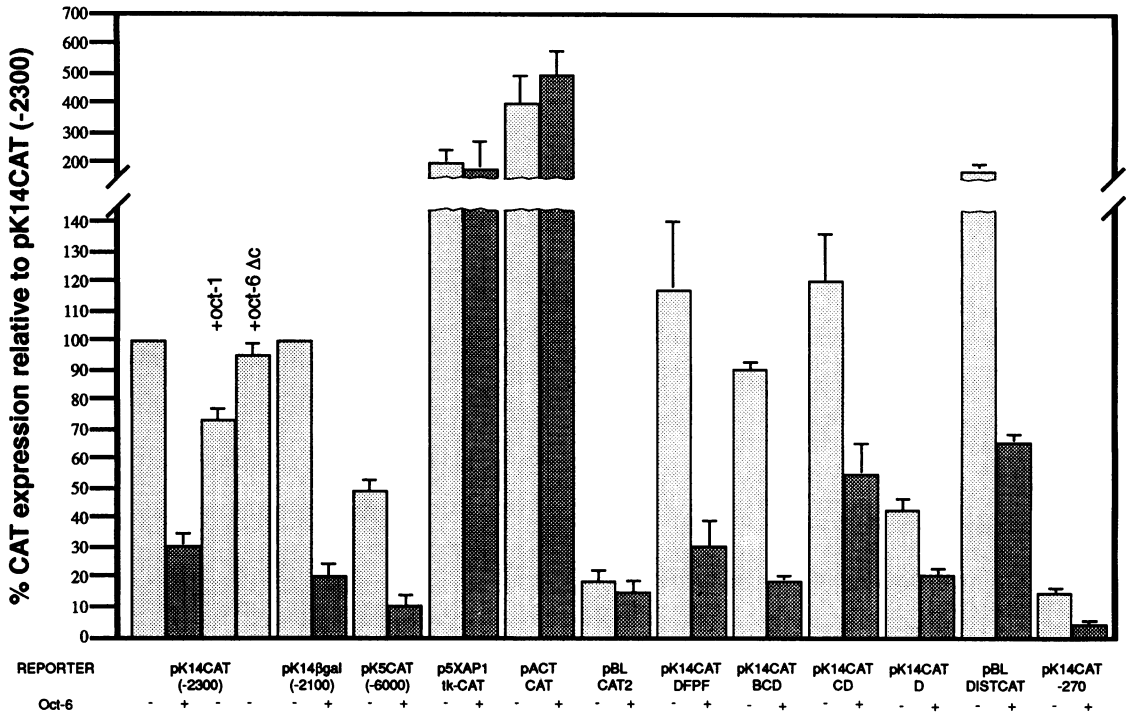

FIG. 9. Effects of human Oct-6 expression on the behavior of human K14 and K5 promoters in cultured human keratinocytes. Constructs shown in Fig. 8 were transiently transfected into SCC-13 keratinocytes, and soluble extracts were prepared 48 h later. A portion of the extract was used for β-Gal assays to adjust samples for variation in transfection efficiencies, which varied from 0.1 to 2.0%. Another portion was used for CAT assays, which were analyzed by ELISA as described in Materials and Methods. CAT levels of each extract were normalized against the control and are expressed as a percentage of the pK14CAT(−2300) value.

containing the 600-bp distal element (~−2300 to −1700) and the 270-bp K14 promoter, cloned in the forward direction 5′ to the CAT gene (Fig. 8). Indeed, Oct-6 repressed expression of pK14CAT(DFPF) to an extent comparable to that of pK14CAT(−2300) (Fig. 8 and 9).

Three additional CAT constructs containing distal elements of reduced sizes still maintained the sensitivity to Oct-6 expression, as did a construct in which the proximal element of the promoter had been replaced by the *tk* promoter (see Fig. 8 for constructs and Fig. 9 for CAT assay data). Surprisingly, a construct containing the proximal element also exhibited repression by Oct-6, suggesting that both proximal and distal elements contain sequences which can be negatively influenced by Oct-6. These tests narrowed the Oct-6 sensitivity to a 168-bp region of the distal element and the 270-bp proximal element of the K14 promoter. These regions are also the portions of the K14 promoter responsible for the bulk of positive activity in cultured keratinocytes. In fact, taking these and additional mutagenesis studies together, we did not find a segment of the K14 promoter that contained appreciable activity but that was not suppressed by Oct-6.

To determine whether Oct-6 exerted its effects on the K14 distal element directly by binding to DNA, we conducted EMSAs on nuclear extracts from COS cells transfected with pSGhOct6 and on extracts from Oct-6 RNA-primed reticulocyte lysates. Under conditions in which binding of recombinant Oct-6 to the canonical octamer motif were easily demonstrated (Fig. 4A), no direct binding of Oct-6 was observed with either a radiolabeled K14 D fragment (−1814 to −1646) or two radiolabeled proximal fragments (−270 to −93 and −93 to +1) (data not shown). Previously, we identified the keratinocyte proteins that bind to these three segments, and none of them are octamer-binding proteins (20a, 22). Moreover, in these K14 segments, there are no appreciable homologies to the known positive Oct-6-binding elements (54) or to a series of Oct-6-binding elements that are candidates for mediating the negative regulatory effects of Oct-6 on the Po myelination gene (14). Thus, if there are one or more specific sequences within the distal or proximal elements that bind Oct-6, they must do so with significantly reduced affinity(ies) over the octamer sequence. Taken together with our CAT data, these data suggest that the repressive effects of Oct-6 on K5 and K14 gene expression in keratinocytes are mediated either directly, but through cooperative binding with another protein, or indirectly, through regulation of a transcription factor which acts on the distal or proximal elements. Further studies will be necessary to more fully elucidate the complex molecular mechanisms involved in this process.

DISCUSSION

Octamer-binding proteins in skin and other stratified squamous epithelia. Expression of POU domain proteins in skin has only recently been appreciated. As we were conducting the present experiments, Anderson et al. (2) reported the isolation of an Oct-2-related cDNA from a rat pituitary library and reported that this class II POU sequence, called Skn-1a/Skn-1i (related or identical to Oct-11 [11]), is expressed primarily in differentiating cells of epidermis and hair follicles. Furthermore, in an adenocarcinoma cell line cotransfected with a Skn-1a expression vector and a human K10-luciferase reporter gene, Skn-1a exhibited a positive effect on luciferase expression, further implicating Skn-1a as a possible regulator of terminal differentiation in keratinocytes (2). Although our

study has focused on Oct-6, we also detected Skn1a/Oct-11-hybridizing sequences in epidermis. Curiously, however, the hybridization that we detected was predominantly basal rather than suprabasal (5a), a finding in agreement with a recent report by Yukawa et al. (58).

In a separate study, Agarwal and Sato (1) reported that XLPOU1, a *Xenopus* class III POU cDNA cloned from an adult brain library, is expressed in adult skin, as judged by Northern (RNA) and in situ hybridization. While the identity of XLPOU1 remains to be established, this protein belongs to the POU III class and shares 92% sequence homology with SCIP/Tst-1/Oct-6 (1). Prior to our study, expression of Oct-6 in mammalian epidermal development and differentiation had not been extensively analyzed or widely appreciated. However, in a study of Tst-1 expression in rat brain, He et al. (14) noted in passing that head skin displayed intense hybridization with a radiolabeled Tst-1 probe. Similarly, although not mentioned in a study of Oct-6 expression in developing mouse brain (45), there was nevertheless appreciable hybridization of an Oct-6 probe to head skin. When taken together with our data, this evidence strengthens the notion that POU III homeodomain proteins are expressed broadly in stratified squamous epithelia, in which they may play a role in development and differentiation.

Class III POU domain proteins and their action in other systems. In accordance with its designation as an octamer-binding protein, Oct-6 binds the motif ATTTGCAT, and it can activate a reporter gene driven by a promoter containing this motif (45). However, Oct-6 is interesting in that it is one of the few POU proteins known to act in both positive and negative fashions, and intriguingly, octamer elements are not always involved in mediating these effects. For example, Oct-6 binds specifically to six GA(A/T)T(A/T)ANA sites in the promoter of a Schwann cell surface adhesion (Po) gene, apparently causing repression (14). Likely relevant is the additional observation that during Schwann cell differentiation, Oct-6 is markedly downregulated (8, 29, 30). In contrast to its effects on Po expression, Oct-6 has a positive effect on both early and late transcription of the glial cell-specific human papovavirus JC genome (54). Only one of the two Oct-6 binding sites in the JC regulatory region is functional, and apart from their A/T richness, the two sites are distinct from each other and from the negative elements (54).

Another feature of Oct-6 expression is its sensitivity to various factors, including cyclic AMP (30), basic fibroblast growth factor (8), and retinoic acid (RA) (45). In F9 teratocarcinoma cells, Oct-6 expression is markedly downregulated upon RA addition (45). Interestingly, although the Pit-1 gene expression is induced by RA, it may be relevant that the Pit-1 protein itself can act as a coregulator of RA receptors (RARs), serving a role in transactivation of an unusual RA response element in the enhancer element of the Pit-1 gene (33). Given that the expression of members of both the POU domain and the RAR family varies in a cell-type- and differentiation-stage-specific manner, it seems possible that this action, if general, may elicit complex modes of action depending on the tissue and the relative DNA binding sites involved. This might be particularly interesting for POU proteins expressed in stratified squamous epithelia, in which the patterns of RAR expression are elaborate (references 26 and 59 and references therein).

Oct-6 and its action on keratinocyte gene expression. One method for identifying the transcription factors governing development and differentiation of a particular cell type is to decipher the mechanisms underlying the regulation of genes encoding proteins unique to the cell. The typifying feature of the mitotically active keratinocyte of stratified squamous epithelia is the expression of K14 and K5 (9, 31). The human genes encoding these two keratins have been cloned and characterized, and a few transcription factors controlling their coexpression have been deciphered. Included in this group are AP2 and Sp1, which bind to both the K5 and K14 promoters and which function in their regulation (reference 5 and references therein). In addition, transcription of the K5 and K14 genes is repressed by RA (42, 49), although it remains unresolved as to whether this regulation is mediated by direct binding of RARs.

In this report, we have found that Oct-6 is expressed significantly throughout all metabolically active layers of stratified squamous epithelia. Moreover, we have shown that overexpression of Oct-6 in cultured keratinocytes results in a dramatic and specific suppression of K5 and K14 gene expression. Thus, Oct-6 is now added to a small but growing list of regulators of epidermal gene expression.

How might Oct-6 exert its effects on epidermal differentiation and keratin gene expression? Since elevated levels of Oct-6 result in K5 and K14 gene suppression, it seems most likely that Oct-6 might play a role in the downregulation of K5 and K14 expression known to occur as epidermal cells commit to terminally differentiate (9, 35). This notion is particularly intriguing given that the regions involved in Oct-6 mediated suppression of the K5 and K14 genes are also those which seem to be involved in positively regulating K5 and K14 gene expression (5, 22). This said, our studies suggest that the mechanism of Oct-6 action is complex. Thus, both proximal and distal elements of the K14 gene seem to be involved in Oct-6 suppression, yet surprisingly, no Oct-6 binding was detected in these regions under conditions sufficient to detect Oct-6 binding to known octamer and nonoctamer elements. This finding is especially ironic considering that the POU homeodomain seems to be required for Oct-6 action. Taken together, our results suggest that either Oct-6 mediates its interaction through a proximal and/or distal element DNA-binding protein or Oct-6 binds to the K14 promoter via additional factors or modifications. In the former scenario, this could either be indirectly, e.g., through influencing transcription of a protein or directly, e.g., through interaction with a protein. As mentioned above, while the behavior of Oct-6ΔC might be suggestive of a mechanism involving DNA binding, we cannot rule out the possibility that action occurs via alternative mechanisms.

One final observation which must be reconciled is that Oct-6 or an Oct-6-related mRNA is expressed not only in differentiating epidermal cells, in which K5 and K14 genes are suppressed, but also in basal cells, in which the K5 and K14 genes are normally expressed. One model to fit these data is that in the basal keratinocyte, there are normally sufficient levels of a factor(s) that prevents the action of Oct-6 on the K14 and K5 genes. During terminal differentiation, the balance of these two factors might be altered, either through downregulation of an activator protein or through upregulation (or modification) of Oct-6, thereby resulting in suppression of K5 and K14. While additional studies will be necessary to distinguish among these possibilities, it is interesting to consider that a basal keratinocyte suppresses K14 and K5 expression not only irreversibly, during terminal differentiation, but also reversibly, during wound healing and other periods of hyperproliferation. Consequently, there may be a greater need for flexibility in K5 and K14 gene expression than previously appreciated, and Oct-6 may be a potential candidate for fulfilling this role.

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