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Transcription factor AP2 and its role in epidermal-specific gene expression

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ABSTRACT **The epidermis is a stratified squamous epithelium whose major differention-specific products are keratins. To elucidate factors controlling keratinocyte-specific gene expression, we previously identified proximal and distal regulatory elements that act synergistically to drive keratinocyte-specific expression of the gene encoding human epidermal keratin K14. Control by the proximal element is mediated by a transcription factor, KER1, which is more abundant in nuclear extracts of keratinocytes than in extracts of other cell types, including fibroblasts, lymphocytes, and simple epithelial cells. In this report, we identify this factor as transcription factor AP2, shown to be transcribed in cells of epidermal and neural crest lineages. Furthermore, we demonstrate functional AP2 binding sites upstream from three additional epidermal genes, suggesting that AP2 may be generally involved in epidermal gene regulation. Finally, although AP2 is necessary, it is not sufficient for epidermal gene expression: a distal element contributes to tissue-specific expression of the human keratin K14 gene as judged by its ability to enhance expression of a heterologous promoter in keratinocytes but not in hepatoma cells. These results imply that a combination of factors is likely to contribute to epidermal-specific expression of the human keratin K14 gene.**

Keratinocytes are surface and lining cells found exclusively in stratified squamous epithelia, including epidermis, tongue, esophagus, cervix, and cornea (for review, see refs. 1 and 2). Mitotically active keratinocytes appear to be morphologically and biochemically similar irrespective of tissue origin (3). The major proteins of these cells are keratins K5 (58 kDa) and K14 (50 kDa), which form an extensive network of 10-nm cytoskeletal filaments (3). As keratinocytes differentiate, they down-regulate this pair of keratins and switch on expression of other pairs, the nature of which is dependent upon tissue origin (1, 2, 4). Terminally differentiating epidermal cells express K1 (67 kDa) and K10 (56.5 kDa) *in vivo* (1, 4), whereas they express K6 (56 kDa) and K16 (48 kDa) *in vitro* (5). Differentiating cells of other stratified squamous epithelia express other pairs (1, 2). Tissue-specific differences in keratin expression are largely exerted at the transcriptional level (6–8).

As judged from other differentiation systems, a knowledge of the factors controlling keratinocyte-specific gene transcription should be important in elucidating the mechanisms underlying commitment of cells to a keratinocyte fate. Sequence comparison of the 5′ upstream regions of keratinocyte genes has revealed several consensus elements found in many epidermal genes. These include the simian virus 40 (SV40) core enhancer sequence (9) and the so-called CK8mer sequence 5′-AANCCAAA-3′, where N is any base (10, 11). However, it has not been demonstrated that these sequences are major contributors to gene expression (10, 12).

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Recently, some transcription factors involved in keratinocyte expression have been identified. A sequence, 5′-ACCCTGAGGCT-3′, located upstream from the "TATA box" of a *Xenopus* keratin gene binds a transcription factor termed KTF1, highly enriched in embryonic epidermal nuclear extracts (13). A similar sequence, 5′-GCCTGCAGGC-3′, located upstream from the TATA box of the human K14 keratin gene binds an epidermal-enriched factor termed KER1 (14). This palindromic sequence acts synergistically with a distal element to drive expression of the human keratin K14 gene in epidermal cells *in vitro* and in transgenic mice *in vivo* (14). Since half the KER1 palindrome resembles a consensus binding site for transcription factor AP2 (15) and since an AP2 oligomer competes effectively for KER1 binding (14), we surmised that this factor may be related to the epidermal and neural crest lineage-specific transcription factor AP2 (14, 16). In this paper, we identify KER1 as AP2, demonstrate the existence of AP2 binding sites in three additional epidermal keratin genes, and assess the involvement of AP2 in keratinocyte-specific gene expression.

MATERIALS AND METHODS

Protein Extracts, Nuclear Extracts, and Gel-Shift and Methylation-Interference Assays. Total and intermediate filament proteins were isolated and analyzed (see ref. 7). Nuclear extracts were prepared and methylation-interference and gel-shift assays were performed as described (14). AP2 cDNA was transformed into a bacterial expression vector and AP2 protein was generated (15). Competitor oligomer bearing a consensus AP2 sequence was obtained from Stratagene.

Cell Culture, Transfections, Chloramphenicol Acetyltransferase (CAT), and β-Galactosidase Assays. SCC13 ($K14^+$), HeLa ($K14^-$), WI38 ($K14^-$), and MCF7 ($K14^-$) cells were cultured as described (14). HepG2 cells were maintained as described (15). Transfections were as described (14). Cells (100-mm plate) were transfected with 3.5 μg of control β-galactosidase plasmid pCH110 (see ref. 14) and an amount of CAT reporter DNA equimolar to 10 μg of the standard test plasmids pK14CAT(−2300) or pBLCAT2 (17). Variable amounts of pKS (Stratagene) carrier DNA were added to 30 μg of total DNA per transfection. Cell extracts, colorimetric ELISA, CAT, and β-galactosidase assays were as described (14).

RESULTS

KER1 and AP2 Are Identical or Closely Related Proteins. Previously, we reported that a double-stranded oligomer containing a consensus AP2 site competed for binding of a

Abbreviations: SV40, simian virus 40; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic-mobility-shift assay; TK, thymidine kinase.

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keratinocyte nuclear factor, KER1, to a radiolabeled 176-base-pair *Bsu*361–*Nhe* I K14 promoter fragment (residues −270 to −94) containing the KER1 binding site (14). Thus, when this K14 fragment was combined with nuclear extract from SCC13 keratinocytes ($K14^+$), a specific complex with KER1 formed (Fig. 1*A*, lane 2). A 100-fold molar excess of unlabeled competitor AP2 oligomer quantitatively blocked formation of this complex (Fig. 1*A*, lane 3; see also ref. 14). This result demonstrated that KER1 could bind to a consensus AP2 sequence in addition to the K14 KER1 palindromic sequence.

To investigate in greater detail the relation between AP2 and KER1, we compared complexes formed between radiolabeled K14 promoter fragment and either KER1, from keratinocyte nuclear extracts, or cloned human AP2, purified from a bacterial expression system. As judged by electrophoretic-mobility-shift assays (EMSAs), the mobility of the AP2–K14 promoter complex (Fig. 1*A*, lane 4) was identical to that of the KER1–K14 complex (Fig. 1*A*, lane 2). Thus, the behavior of AP2 and KER1 was indistinguishable by EMSA.

To determine whether KER1 and AP2 were related antigenically, we incubated our keratinocyte nuclear extracts with either bovine serum albumin (control) or anti-AP2 antiserum (test) prior to combining the extract with radiolabeled K14 promoter fragment (Fig. 1*B*, lanes 1 and 2, respectively). Preincubation of the nuclear extract with anti-AP2 antiserum resulted in a DNA–protein complex (solid arrow) that exhibited an electrophoretic mobility that was significantly retarded when compared with the extract alone (solid arrowhead). The ability of anti-AP2 antibodies to retard migration of the KER1–DNA complex indicated that KER1 and AP2 share antigenic epitopes, suggesting that they are related or identical proteins.

Previously, we utilized a methylation-interference assay to characterize the K14 promoter DNA binding site of the KER1 protein (14). To determine whether AP2 and KER1 recognized the same nucleotides in this site, we repeated these assays, this time using bacterially expressed human AP2 protein. Both coding and noncoding strands were analyzed. Methylation of coding-strand guanine residues at positions −231, −227, −224, and −223 interfered strongly with cloned AP2 binding (Fig. 2*A*). Methylation at guanine residues −230, −229, and −226 on the noncoding strand also interfered strongly with AP2 binding (Fig. 2*A*). This pattern of interference was indistinguishable from that obtained with KER1-containing nuclear extracts (Fig. 2*B*), suggesting that KER1 is AP2.

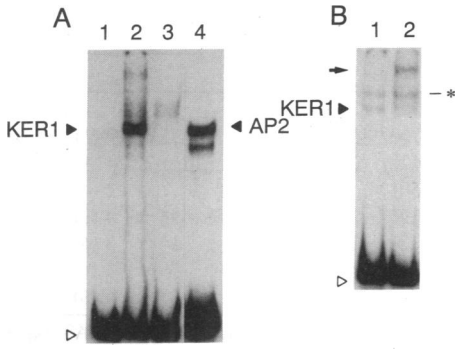

FIG. 1. Identification of KER1 as AP2 by competition and antibody analyses (EMSAs). (*A*) Competition between KER1 and AP2. Radiolabeled probe was the human K14 promoter (10,000 cpm, 0.5 ng) containing the KER1 binding site. Lanes: 1, probe incubated with no protein; 2, probe incubated with SCC13 nuclear extract; 3, probe incubated with SCC13 extract and a 100-fold molar excess of competitor oligomer bearing a consensus AP2 site; 4, probe incubated with cloned AP2 protein. (*B*) Demonstration of shared epitopes between KER1 and AP2. Lanes: 1, K14 probe combined with SCC13 extract preincubated with 5 μg of bovine serum albumin; 2, K14 probe combined with SCC13 extract preincubated with 1 μl of anti-AP2 antibody. Solid arrowheads in *A* and *B* denote KER1 or AP2 DNA complexes. Open arrowheads denote free DNA. Arrow in *B* denotes retarded KER1–anti-AP2 antibody–DNA complex. Asterisk in *B* denotes nonspecific band (see ref. 14).

AP2 Binds the Promoters of Other Epidermal Keratinocyte Genes. If AP2 plays a general role in keratinocyte-specific gene expression, it might be expected to bind other epidermal promoters. Previously, we noted the sequence 5′-CCCCCAGGC-3′, originally thought to be the consensus sequence for AP2 (15), at positions −100 to −92 upstream of the human K5 gene transcription initiation site (Fig. 3*A*; ref. 6). To determine whether AP2 binds to this sequence, we combined purified cloned AP2 with a radiolabeled 89-base-pair *Sac*I–*Hph* I K5 promoter fragment (residues −111 to −22) containing the putative AP2 site. A complex formed that could be resolved by acrylamide gel electrophoresis (Fig. 3*B*, compare lane 1, no protein, with lane 2, added AP2 protein). This binding was specific, as complex formation was inhibited by adding a 100-fold molar excess of either an oligomer bearing a consensus AP2 site (lane 3) or the unlabeled probe (lane 4).

In our earlier studies (14), we noted that a DNA sequence, 5′-GGCTGCAGGC-3′, nucleotides −122 to −113 upstream from the transcription initiation site of the K1 gene, was similar to the KER1 binding site of the human K14 gene (Fig. 3*A*; refs. 14 and 19). Inspection of the human K6b gene (Fig. 3*A*; ref. 20) revealed another KER1/AP2-like site, 5′-GCTGGAGGC-3′, nucleotides −329 to −321 upstream from this gene's putative transcription initiation site. To determine whether these sequences bound AP2, we tested the ability of double-stranded oligomers to compete with the K14 pro-

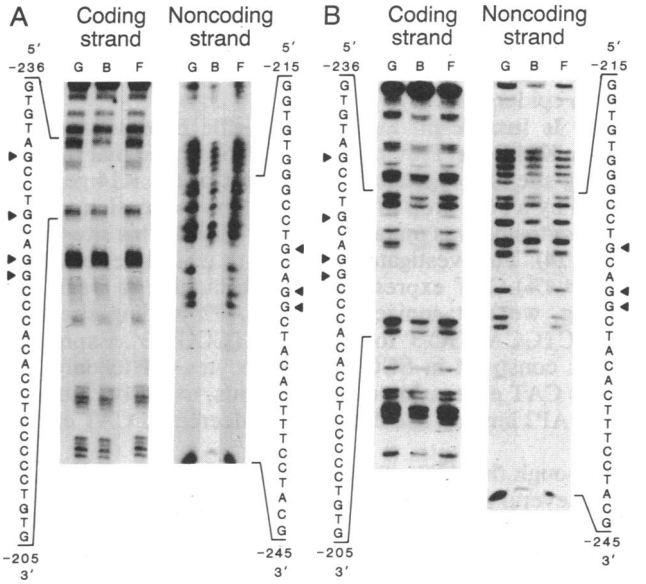

FIG. 2. Identification of KER1 as AP2 by methylation-interference analysis. K14 probe DNA was partially methylated with dimethyl sulfate prior to combining with cloned human AP2 protein (*A*) or with SCC13 nuclear extract (KER1) (*B*). DNA–protein complexes were separated from free DNA by using EMSA. Complexes were eluted, cleaved with piperidine, and analyzed on sequencing gels. Results for coding and noncoding strands are shown. Lanes: G, sequence pattern of partially methylated DNA probe chemically cleaved at guanine residues; B, analogous sequence pattern, but of that portion of the partially methylated DNA probe that bound KER1 or AP2 protein; F, analogous sequence pattern, but of that portion of the partially methylated DNA probe that did not bind KER1 or AP2. Solid triangles denote residues that, when methylated, strongly interfered with factor binding.

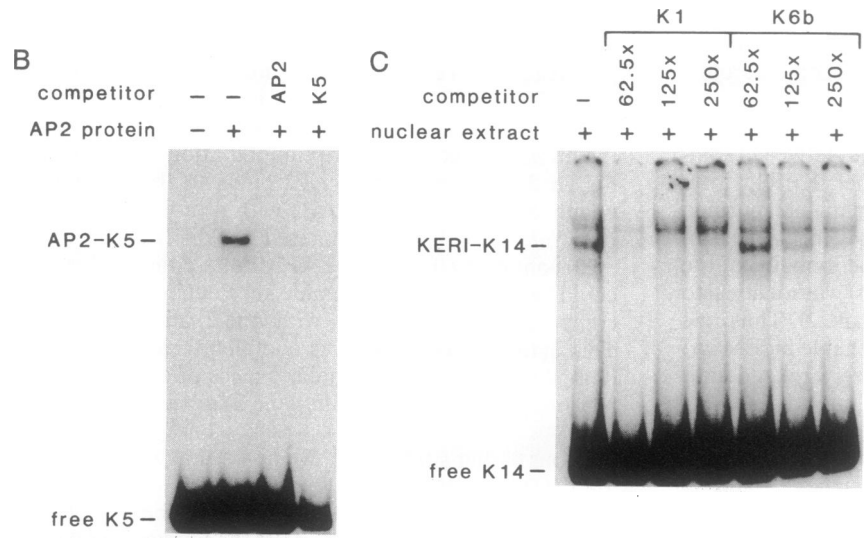

FIG. 3. Binding of AP2 to other epidermal keratin promoters. (*A*) AP2-like sequences in human epidermal keratin promoters. Sequences listed are as follows: AP2 (con 1), the AP2 consensus sequence (18); AP2 (con2), the AP2 consensus sequence (15); K14, human K14 gene (9); K5, human K5 gene (6); K1, human K1 gene (19); K6b, human K6b gene (20). (*B*) Binding of AP2 to the K5 promoter. Radiolabeled K5 probe was used in an EMSA with purified cloned AP2 protein. Competitors were either a 100-fold molar excess of oligomer bearing a consensus AP2 site or unlabeled K5 probe, as indicated. (*C*) Binding of KER1 to radiolabeled K14 promoter in the presence of increasing amounts of unlabeled oligomers harboring human K1 and human K6b AP2 sites.

moter for the binding of AP2 (KER1) in keratinocyte nuclear extracts. Both K1 and K6b sequences competed for AP2 binding at 250-fold molar excess (Fig. 3*C*). However, at 62.5- and 125-fold molar excesses, the K1 sequence competed more effectively than K6b, indicating a higher affinity for AP2. Collectively, our binding studies showed that several epidermal genes have AP2 binding sites within the first several hundred nucleotides upstream from their transcription initiation sites. This opens the possibility that AP2 may play a widespread role in regulating gene expression in human epidermis.

AP2 Is Insufficient for Tissue-Specific Expression of the Human K14 Gene. Previously, we demonstrated that the AP2 binding site is a regulatory component of the K14 gene, as judged by a 56% drop in K14–CAT gene expression when a single cytosine was mutated to an adenine in this site (Table 1; ref. 14). To investigate the extent to which the remaining 44% (±4%) CAT expression was attributable to weak AP2 binding, we mutagenized the K14 AP2 binding site from 5'-GCCTGCAGGC-3' to 5'-GAGGATCCGC-3'. Expression of this construct in SCC13 keratinocytes led to only 14% (±8%) CAT expression (Table 1). Thus, extensive mutation of the AP2 binding site dramatically decreased CAT expression.

Although the AP2 site was involved in K14 gene expression, several lines of evidence suggested that AP2 might not be sufficient for K14 expression. Perhaps the best evidence was the finding that a distal element acting synergistically with the K14 proximal element was necessary for appreciable expression in both cultured keratinocytes and transgenic mice (14). Although the factors interacting with this distal element remain to be identified, the element did not compete for binding of AP2 to the proximal element (14). To more carefully assess the extent to which factors in addition to AP2 might be required for keratinocyte-specific expression, we examined a variety of cell lines for (*i*) the presence of AP2 in their nuclear extracts, (*ii*) their ability to express transiently transfected pK14CAT(−2300), and (*iii*) expression of endogenous K14 protein.

Both normal and SCC13 epidermal cells expressed considerable levels of AP2 (Fig. 4*A*, lanes 1 and 2, respectively). Normal keratinocytes did not transfect well, but SCC13 keratinocytes expressed high levels of CAT when transfected with pK14CAT(−2300) (Fig. 4*B*). Normal human epidermal and SCC13 cells also expressed appreciable levels of endogenous K14 as shown by total protein and intermediate filament extracts (see Fig. 4*C*). Thus, for keratinocytes, a good correlation existed for AP2 expression, K14–CAT expression, and endogenous K14 expression. Despite these correlations, the level of K14 in normal keratinocytes was 5–10 times the level in SCC13 cells (compare equal loadings of total protein extracts from these cells in Fig. 4*C*), and this was not reflected at the level of AP2 binding activity in the

Table 1. Expression of K14–CAT constructs in SCC13 and HepG2 Cells

Clone	CAT expression, % SCC13 level	
	SCC13	HepG2
pK14CAT(−2300)	100	4 ± 3 (5)
pK14CAT(−1700/−270)	95 ± 9 (3)	4 ± 3 (3)
pK14CAT(−1120/−270)	111 ± 24 (10)	ND
pK14CAT(−450)	<2 (4)	ND
pK14CAT(−1120/−270 mut −230)	44 ± 4 (2)	ND
pK14CAT(−1120/−270 mut AP2)	14 ± 8 (4)	ND
pBLCAT2	9 ± 3	17 ± 2
pBLDISTCAT	38 ± 5 (6)	6 ± 2 (5)

See Fig. 4 for assays and notations. The 100% CAT expression was assigned as the level obtained by transient transfection of pK14CAT(−2300) into SCC13 cells. Plasmid pK14CAT(−2300) contains 2300 base pairs of sequence 5' from the human K14 gene transcription initiation site linked to the CAT reporter gene. This K14 sequence is sufficient for reporter gene expression in keratinocytes *in vitro* and *in vivo* (21). Plasmid pK14CAT (−X/−Y) contains a deletion in pK14CAT(−2300) between −X and −Y. Plasmids pK14CAT(−1120/−270 mut −230) and pK14CAT(−1120/−270 mut AP2) contain mutated AP2 sites as described in the text. Expression from the TK promoter-driven CAT plasmid pBLCAT2 was used as a reference to measure pBLDISTCAT expression. Plasmid pBLDISTCAT contains the K14 distal element (positions −2300 to −1700) cloned into pBLCAT2 upstream of the TK promoter (positions −105 to +51 of the TK gene; ref. 17). Numbers of independent trials are in parentheses. Control transfections were always conducted with each assay.

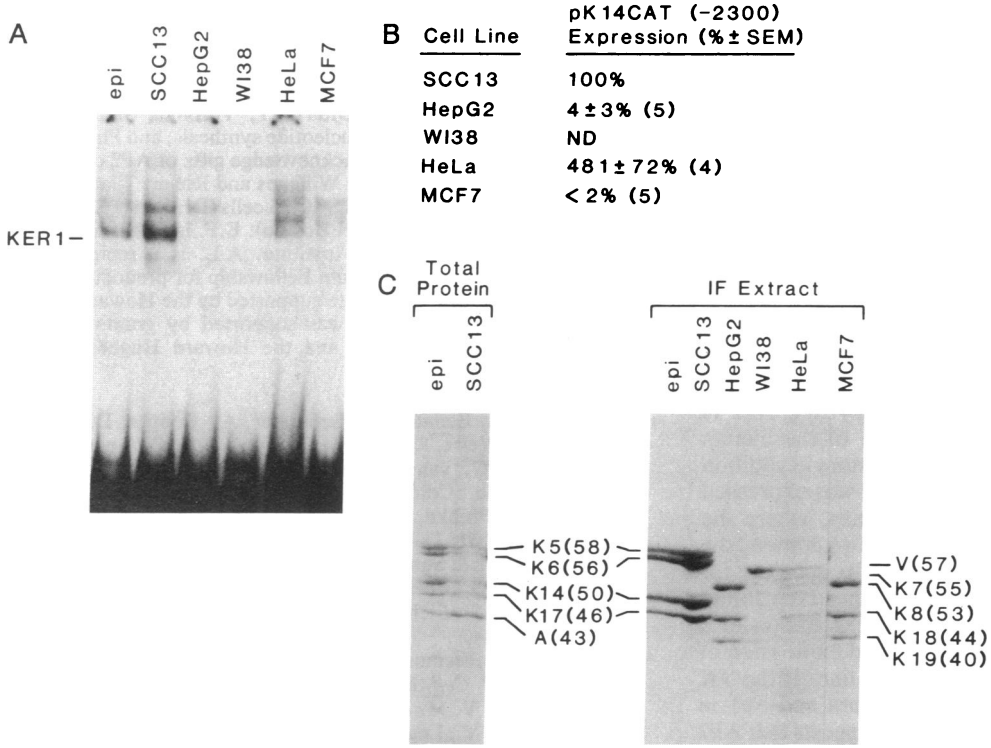

FIG. 4. AP2 is not sufficient to ensure abundant K14 gene expression. (*A*) Tissue specificity of AP2 binding activity. EMSAs were performed by combining radiolabeled K14 promoter with equal amounts (5 μg) of nuclear extracts from epidermal cells (epi), SCC13 keratinocytes, HepG2 hepatoma cells, WI38 lung fibroblasts, HeLa cervical carcinoma cells, or MCF7 breast adenocarcinomal cells. (*B*) Expression of pK14CAT(−2300) transfected into various cell lines. CAT expression is shown as a percentage of that obtained in SCC13 cells. Numbers of independent trials are in parentheses. ND, not determined. (*C*) Expression of endogenous K14 in various cell lines. Total protein and intermediate filament extracts from human epidermal (epi), SCC13, HepG2, WI38, HeLa, and MCF7 cells were resolved by SDS/PAGE, followed by staining with Coomassie blue. K, keratins according to nomenclature of Moll *et al.* (2); V, vimentin; A, actin. Molecular masses in parentheses are in kDa.

two keratinocyte types (Fig. 4*A*, compare lanes 1 and 2). Differences in K14 expression levels could not be attributed to posttranscriptional differences, since K14 transcript levels are much higher in normal epidermal cells than in SCC13 keratinocytes (8). However, if AP2 levels decrease during terminal differentiation, then this could interfere with direct comparisons, since the ratio of differentiating to mitotically active cells is higher in normal than in SCC13 keratinocytes. Alternatively, factors in addition to AP2 may account for differences in K14 levels in keratinocytes.

In nonkeratinocyte lines, there were both correlations and inconsistencies for AP2, K14 transgene, and endogenous K14 gene expression. Thus, for example, the absence of AP2 in the hepatoma cell line HepG2 (Fig. 4*A*, lane 3; ref. 15) correlated well with both lack of expression of pK14CAT(−2300) in these cells (4 ± 3% SCC13 levels; see Fig. 4*B*) and lack of endogenous K14 (Fig. 4*C*, lane 5). Similarly, WI38 lung fibroblasts lacked both AP2 (Fig. 4*A*, lane 4; ref. 14) and endogenous K14 (Fig. 4*C*, lane 6; ref. 7). In contrast, the cervical carcinoma cell line HeLa and the breast adenocarcinoma cell line MCF7 expressed AP2 (Fig. 4*A*, lanes 5 and 6, respectively; refs. 14–16) but did not express endogenous K14 (Fig. 4*C*, lanes 7 and 8; ref. 7). Since nuclear run-off experiments indicate that the K14 gene is not transcribed in MCF7, WI38, and HeLa cells (8), these differences could not be explained by posttranscriptional mechanisms. Furthermore, whereas promiscuous expression (481% SCC13 levels) of K14–CAT was seen in transfected HeLa cells (Fig. 4*B*), only background levels of CAT expression (<2% SCC13 levels) were obtained when MCF7 cells were transfected with pK14CAT(−2300) (Fig. 4*B*). Hence, even though appreciable K14 gene expression depended upon AP2, the mere presence of AP2 did not guarantee expression of the K14 gene.

The Distal Element Contributes to Tissue-Specific Expression of the K14 Gene. To assess whether AP2 is required for tissue-specific expression, we tested the possibility that the K14 distal element (positions −2300 to −1700) might be able to function with a heterologous AP2-less promoter in keratinocytes. The thymidine kinase (TK) promoter–CAT plasmid pBLCAT2 was chosen for analysis, since this promoter is well-characterized, consisting of binding sites for transcription factor TFIID, Sp1, and "CCAAT box" factors (17). We inserted the K14 distal element 5′ upstream from the TK promoter to produce the plasmid pBLDISTCAT. When transfected into SCC13 keratinocytes, pBLDISTCAT elicited CAT expression at a significantly higher level than was obtained with pBLCAT2 (Table 1). Interestingly, pBLDISTCAT-transfected HepG2 cells showed reduced CAT expression over that obtained with pBLCAT2 (see Table 1). Thus, as judged from these data, the distal element not only contributed to keratinocyte-specific enhancement of an AP2-less promoter but also seemed to play a role in promoter repression in a nonkeratinocyte (HepG2) cell line. These studies provide the most direct evidence to date that the K14 distal element not only is required for appreciable levels of gene expression but also is involved in tissue specificity. Additional experiments should reveal distal element transcription factors.

DISCUSSION

Human keratin K14 is a faithful and abundant marker of proliferating basal cells of epidermis and other stratified squamous epithelia (3, 4). Moreover, the tissue specificity of K14 protein expression correlates well with cells in which this gene is transcribed (6, 8, 21). As such, the human K14 gene serves as an excellent model with which to examine the sequences and transcription factors involved in keratinocyte-specific gene expression.

We have identified a factor (KER1) present in SCC13 keratinocyte nuclear extracts that bound to a K14 promoter proximal element involved in keratinocyte-specific transcription (14). Our previous studies (14) suggested that KER1 might be AP2, and we now provide strong evidence to support this hypothesis. We cannot say whether a related factor KTF1 (13) is the *Xenopus* equivalent of AP2, since an oligomer containing the KER1-related KTF1 binding site did not compete at a 50–100 times excess for binding to our K14 promoter fragment (14). However, the strong similarities between the two sites is striking, and the failure to compete

might be due to relative differences in AP2 binding affinities (14).

Several lines of evidence have led to the hypothesis that AP2 might be involved in epidermal gene regulation. Perhaps most compelling was the finding that a gene for human epidermal keratin K14 bound an AP2-like factor (KER1) that positively influenced keratinocyte-specific gene expression (14). In addition, AP2 binding sites were noted in the upstream sequences of several other epidermally expressed genes, including those encoding keratins K5 and K1 (14). More recently, AP2 mRNAs were localized predominantly in cells of neural and epidermal lineages (16). Finally, it was intriguing that SV40 tumor antigen competed for AP2 binding sites (22), since SV40 transformation of keratinocytes has been associated with a decrease in keratin expression (23).

Our present data demonstrate convincingly that AP2 is involved in keratinocyte-specific expression of the human K14 gene. However, they also suggest that factors in addition to AP2 play a role in this process. Thus, AP2 was expressed in some cells, such as HeLa and MCF7 lines, where the endogenous K14 gene was not transcribed. In addition, the K14 distal element, which does not appear to compete for binding of AP2 to the proximal element, enhanced the ability of a heterologous (TK) promoter to drive expression of a reporter gene in keratinocytes but suppressed expression of the transgene in a nonkeratinocyte (HepG2) line. If the TK promoter is regulated by CCAAT box factors and Sp1 in keratinocytes as it is in HeLa cells (24), this suggests that AP2 might be dispensable in driving keratinocyte-specific gene expression, at least *in vitro*. That keratinocyte-specific gene expression involves factors in addition to AP2 is supported by the finding that AP2 mRNAs are expressed in cells of neural as well as epidermal lineages (16). In addition, AP2 mRNA levels can be elevated in response to retinoic acid (25), an agent known to repress K14 gene transcription (8, 26). Thus, although AP2 seems to be involved in gene expression in epidermal cells, it has become increasingly apparent that one or more additional factors will play a role in this process.

It was interesting that the construct pK14CAT(−2300) was promiscuously expressed when transiently transfected into some cell lines (e.g., HeLa) that did not express endogenous K14. This did not seem to be due to the bacterial vector sequences flanking the K14 transgene, since removal of these sequences did not eliminate promiscuous expression (21). It could be that these cells possess transcription factors necessary for expression of the naked K14 gene but that the endogenous K14 promoter is in a conformational state that renders it inaccessible to these factors. Inaccessibility could arise, for instance, by either methylation or chromatin assembly (for example, see ref. 27). If so, transcriptional control might be bypassed by transient transfection of the naked pK14CAT(−2300) plasmid DNA. Precedence for involvement of methylation in keratin gene expression has been provided by Oshima *et al.* (28). In addition, treatment of SV40-transformed fibroblasts with 5-azacytidine induces keratin gene expression (29). Although additional experiments will be necessary to elucidate the molecular basis for promiscuity in expression of the K14–CAT gene, our observations suggest that expression of the human K14 gene may rely, at one level, on the synergistic or antagonistic interactions between AP2 and other nuclear factors and, perhaps, at another level, on the methylation or conformational state of the K14 gene. As further studies are conducted, the complex mechanisms regulating keratinocyte-specific gene expression should become more apparent.

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