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Epithelial-specific keratin gene expression: identification of a 300 base-pair controlling segment

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ABSTRACT

To elucidate the elements required for regulation of keratin expression in epidermis, we have linked a short, 300 base pair segment, corresponding to the promoter region of a human K # 14 gene, to the chloramphenicol-acetyl-transferase gene. This construct was introduced into various mammalian cell lines and primary cultures via $Ca_3(PO_4)_2$ precipitation. The 300 base pair segment from the keratin gene promoter region was active in all epithelial cells studied including transformed, simple epithelial cells such as HeLa and ME-180, cell lines derived from stratified epithelium, such as SCC-12, as well as primary cultures of epithelial cells. The construct was inactive in all non-epithelial cells tested including fibroblasts and melanocytes. The segment does not function as a silencer in nonepithelial cells but it can function as an enhancer in epithelial cells. Using the polymerase chain reaction we have constructed a series of deletions of the promoter and have localized an essential function within a 40 bp sequence. We conclude that we have identified the keratin gene promoter that is sufficient to confer epithelial-specific expression.

INTRODUCTION

The expression of keratins, the intermediate filament proteins of mammalian epithelial cells, is strictly regulated during mammalian development and differentiation (for review and nomenclature see 1,2). Among the earliest markers of embryonic differentiation are keratins K # 8 and K # 18 (3,4), which persist in trophoblasts and the periderm, but are replaced in the embryonic epidermis by the K # 5 and K # 14 keratin pair (5). This pair is expressed throughout adult life but is restricted to the basal layers of all stratified epithelia (6). Suprabasal cells express differentiation-specific keratin pairs. For instance, keratins K # 1 and K # 10 are specific for keratinizing epithelia, while K # 3 and K # 12 are specific for cornea, etc. (2,7,8). Regulation of keratin gene expression is modified by vitamin A and pathologic states (9,10).

The complex patterns of regulation of keratin protein synthesis imply that each keratin gene responds to several independent or possibly, interrelated mechanisms which control its expression. For most genes, including keratin genes, expression is regulated primarily at the level of transcription (9,11–15). But, a controversy exists regarding the molecular mechanisms of the regulation of keratin gene transcription. This regulation generally involves functional binding of trans-acting proteins to the important cis-acting regulatory DNA segments found, most commonly, immediately upstream from the genes. Yet, Vassar et al. (16) failed to observe any specificity conferred by the upstream region of the human K # 14 keratin gene in transfection experiments, although specific expression of the same DNA in the basal layers was seen in a transgenic mouse. Contradicting results were obtained by Blessing et al. (17) who found an enhancer specific for cells of stratified epithelial origin in the proximal upstream region of the bovine K # 6 keratin gene.

In order to analyze the regulatory mechanisms controlling keratin gene expression and attempt to resolve the controversy described above, we have cloned a reporter gene for chloramphenicol-acetyl-transferase (CAT) into a position downstream from the region corresponding to the promoter of a human K # 14 keratin pseudogene. While the sequence of this promoter region is virtually identical to the corresponding sequence of the functional gene (Fig. 1), we do not presently know whether the differences between the sequences have any effect on the promoter. The protein coding region of the pseudogene has suffered several deleterious mutations (18). In this manuscript we report that 300 base pairs of this promoter region are sufficient to confer epithelial-specific expression on the reporter gene in transient expression assays.

MATERIALS AND METHODS

Gene Constructs

Constructs pSV2-CAT, pRSVZ, and the genomic keratin clone 2H3 have been previously described (18–20). We used standard DNA manipulation procedures for engineering of the DNA constructs (21). The 300 base pair Pst I fragment from a human

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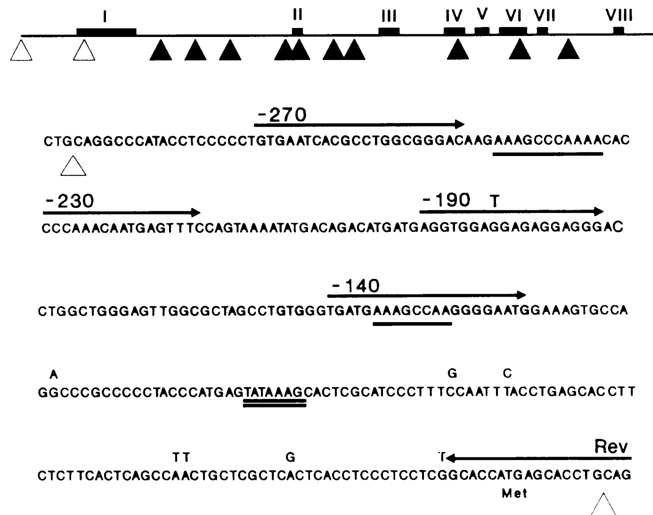

Figure 1: Sequence of the K # 14 keratin pseudogene upstream region. The top line represents the structure of the gene: the bars with Roman numerals represent the exons, the triangles represent the Pst I sites. The empty triangles mark the Pst I sites used to construct the pK14-CAT plasmid. The nucleotide differences present in the functional gene are shown above the sequence. Horizontal arrows mark the primer sequences used in the polymerase chain reaction. ESC segments are underlined, the TATA box is doubly underlined and the initiation codon, which is at position +1, is marked with Met.

genomic clone 2H3 (Fig. 1) was cloned into the Pst I site of M13MP19 vector and its sequence was confirmed by the chain termination method (22). Utilizing the vector's multiple cloning sites, we excised the insert by digestion with Eco RI and Hin dIII. The CAT gene was excised from the plasmid pRSV-CAT by digestion with Hin dIII and Bam HI. The two excised DNA fragments were ligated to the pUC9 vector digested with Eco RI and Bam HI, resulting in the plasmid pK14-CAT. Promoterless control plasmid pUC-CAT was obtained by inserting the Bam HI-Hin dIII fragment containing the CAT gene into pUC9.

Deletions of the 300 bp fragment were constructed using the polymerase chain reaction (23). DNA primers were made on the Parmacia Gene-Plus synthesizer. The same reverse-orientation primer, CGCTGCAGGTGCTCATGGTGC (Rev in Fig 1), was amplified separately with four forward-oriented primers: CGCTGCAGTGAATCACGCCTGGCGGGAC (Primer −270 in Fig 1, these numbers are relative to the initiating codon); CCCTGCAGCCCCAAACAATGAGTTT(−230); CGCTGCAGGTGGAGGAGAGGAGGG(−190); and CGCTGCAGTGGCGCTAGCCTGTGGG (−140). Each primer has a Pst I site and two additional nucleotides at its 5′ end to facilitate cloning into pUC19 vector. Concerned with the fidelity of the polymerase chain reaction (24), we confirmed the sequences of all amplified DNAs using the di-deoxy plasmid sequencing method (25).

The ends of the Eco RI/Hin dIII fragment from pK14-CAT were filled in with Klenow DNA polymerase and ligated into similarly treated Bam HI-digested pSV2-CAT to create plasmid pSV2-CAT-K14. The Bam HI-Bgl II fragment from plasmid pA10CAT, contains the promoter but not the enhancer of the SV40 virus linked to CAT (26). It was inserted into the Bam HI sites of pK14-CAT and pK14-140 to create the plasmids described in Fig. 4b. Their structures were ascertained by restriction analysis.

Cell Maintenance and Growth

Human epithelial cell lines HeLa, ME-180, MCF-7, and SCC-12, as well as murine 3T3 and human GM10 fibroblast lines were a gift from T.-T. Sun. SK-MEL28 human melanoma cell line was obtained from R. Harning. The squamous cell carcinoma cell line SCC-12 was grown in the presence of mitomycin-treated murine 3T3 fibroblast feeder cells as described (27). SCC-12 medium contains DMEM and F_{12} media in a 1:1 ratio, supplemented with 10% fetal calf serum (FCS), and 5 ng/ml hydrocortisone. After mitomycin treatment, fibroblasts do not express any CAT activity when transformed with functional CAT constructs (data not shown). 3T3 and HeLa cells were maintained in Dulbecco-modified Eagle's medium supplemented with 10% calf serum. SK-MEL28 cells were maintained in Eagle's medium supplemented with 20% fetal calf serum, 1% nonessential amino acids and Fungizone. All other established cell lines were maintained in DMEM supplemented with 10% FCS. All media contained penicillin and streptomycin. Cells were grown at 37°C in a 5% CO_2 atmosphere.

Primary cultures of rabbit corneal epithelial cells, esophageal epithelial cells and dermal fibroblasts were derived as described (2) and maintained in DMEM with 20% FCS and 5 ng/ml of hydrocortisone in 60 mm^2 dishes until they were approximately 60% confluent. The two epithelial cell types were grown in the presence of a 3T3 fibroblast feeder layer, as described (7).

Cell Transformation

We have generally followed the procedure of Chen and Okayama (28). One day before transformation, cells were plated onto 100 mm Falcon dishes at the optimal density which produces a near-confluent culture when the cells are harvested. The medium was changed 4 hrs before transformation. Into each dish, the indicated amount of the CAT plasmid, 2.5 μg pRSVZ reference plasmid and a sufficient amount of carrier to bring the total to 20 μg of DNA were added. Forty-eight hours after transfection, the cells were harvested by scraping and subjected to sonication and several freeze-thaw cycles.

Since the DNAs were introduced into various cell types in a large number of separate experiments, we were particularly concerned with reproducibility and quantitative accuracy of our results. All DNA samples were purified by two CsCl-ethidium bromide equilibrium banding centrifugations. For each construct, we used at least three different DNA concentrations and ascertained that the results of the CAT assay were linear relative to the amount of transforming DNA. In each experiment the CAT construct was co-transformed with pRSVZ plasmid (20) so that the CAT and the beta-galactosidase (B-GAL) activities could be determined on the same sample of cell extract.

The B-GAL activity serves as the internal control to monitor transfection efficiency for each dish. It was assayed as described (20). For CAT assays, 50 μl of each extract was heated to 65°C for 10 min, clarified by centrifugation for 10 min, and used as described by Gorman et al. (19). After silica gel chromatography and autoradiography, the spots were excised from the thin-layer plates and counted. The numbers were normalized for transfection efficiency within a given cell type by calculating the ratio of CAT activity to B-GAL activity in each transfected plate.

Primary cultures were transformed and assayed similarly except that they were not passaged before transfection. Indicated amounts of CAT plasmid and 1.25 μg of pRSVZ control were used in corneal and esophageal cells and each experiment was repeated

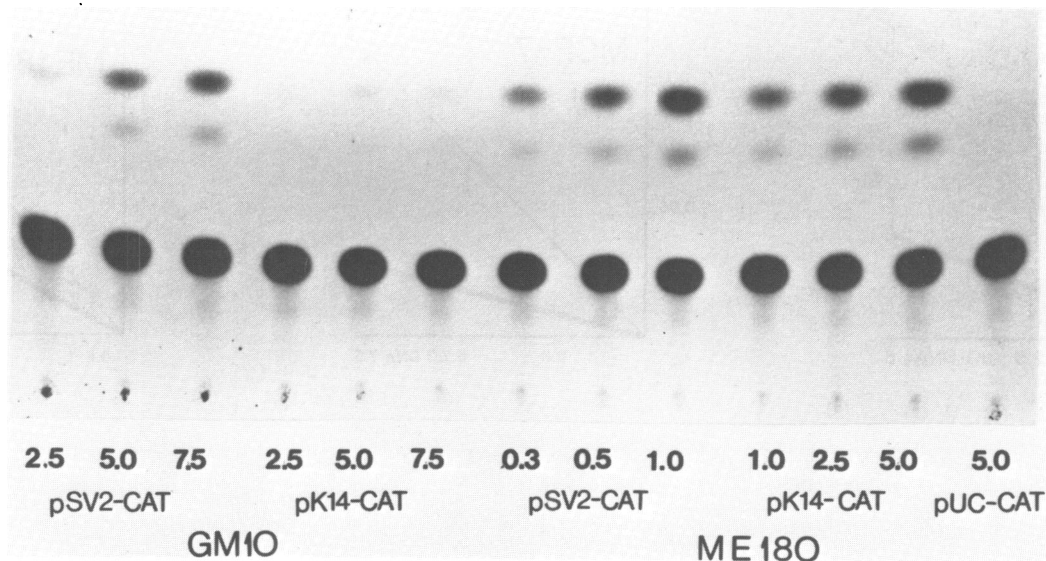

Figure 2: CAT activities in transfected GM10 fibroblasts and in ME-180 epithelial cells. We used 30 μl of extract of GM 10 and 10 μl of ME-180 cells. Numbers beneath each track indicate amounts of input DNA.

Table I. pK # 14-CAT EXPRESSION IS EPITHELIA-SPECIFIC

Cells	Type	Epithelial	Expresses (6) K # 14 Keratin	pUC-CAT	pSV2-CAT	pK14-CAT	$\frac{\text{pK14-CAT}}{\text{pSV2-CAT}}$ (×100)
ME-180	Epidermoid carcinoma	yes	yes	0.2	576 (151)	330 (58)	58
SCC-12	Squamous cell carcinoma	yes	yes	0.1	32 (7)	9.5 (3)	30
HeLa	Cervical adenocarcinoma	yes	no	0.6	134 (23)	58.5 (31)	44
MCF-7	Mammary adenocarcinoma	yes	no	0.1	21 (4)	3.6 (0.6)	18
GM10	Fibroblast	no	no	0.1	30 (18)	0.7 (0.4)	2
3T3	Fibroblast	no	no	0.1	3.1 (0.8)	0.1 (0.04)	3
SK-ME128	Melanoma	no	no	0.1	616 (44)	0.3 (0.1)	0.05
Rabbit cornea	Epithelial	yes	yes	0.02	99 (20)	201 (38)	203
Rabbit esophagus	Epithelial	yes	yes	0.1	212 (16)	310 (36)	148
Rabbit skin	Fibroblast	no	no	0.1	653 (240)	76 (49)	11

The CAT activities were divided by the corresponding B-gal activities, as described in Materials and Methods, and multiplied by 1000. The numbers in parentheses indicate standard deviations.

in duplicate with primary cultures initiated from three or more different rabbits.

RESULTS

Plasmid pK # 14-CAT contains 300 bp of DNA from the 5′ upstream region of the human K # 14 keratin pseudogene in front of the CAT reporter gene. To show that the 300 bp segment contains a functional promoter, the plasmid was introduced into cultured epithelial cells, where it directed expression of the CAT gene (Fig. 2). This proves that the 300 bp segment contains a functional promoter. Furthermore, the promoter activity of pK14-CAT is epithelial-specific: activity was very close to background in the non-epithelial, fibroblast and melanocyte cell lines, but was significantly higher in all cell lines of epithelial origin (Table I, Fig. 3).

Having clearly established the epithelial specificity of the K # 14 promoter transcription in immortalized cell lines, we confirmed this specificity in primary cultures. We decided on two epithelial cell types, cornea and esophagus, both of which express relatively high amounts of K # 14 keratin protein. Primary dermal fibroblasts served as a non-epithelial control. For consistency all three cell types were initiated from the same organism, rabbit, *Orycytolagus cuniculus*.

Primary epithelial cultures were plated in the presence of 3T3 cells and were transformed without passaging when they reached approximately 60–75% confluency. The primary fibroblasts were transformed at the same confluency. The keratin promoter was highly active in both epithelial cells and was even stronger

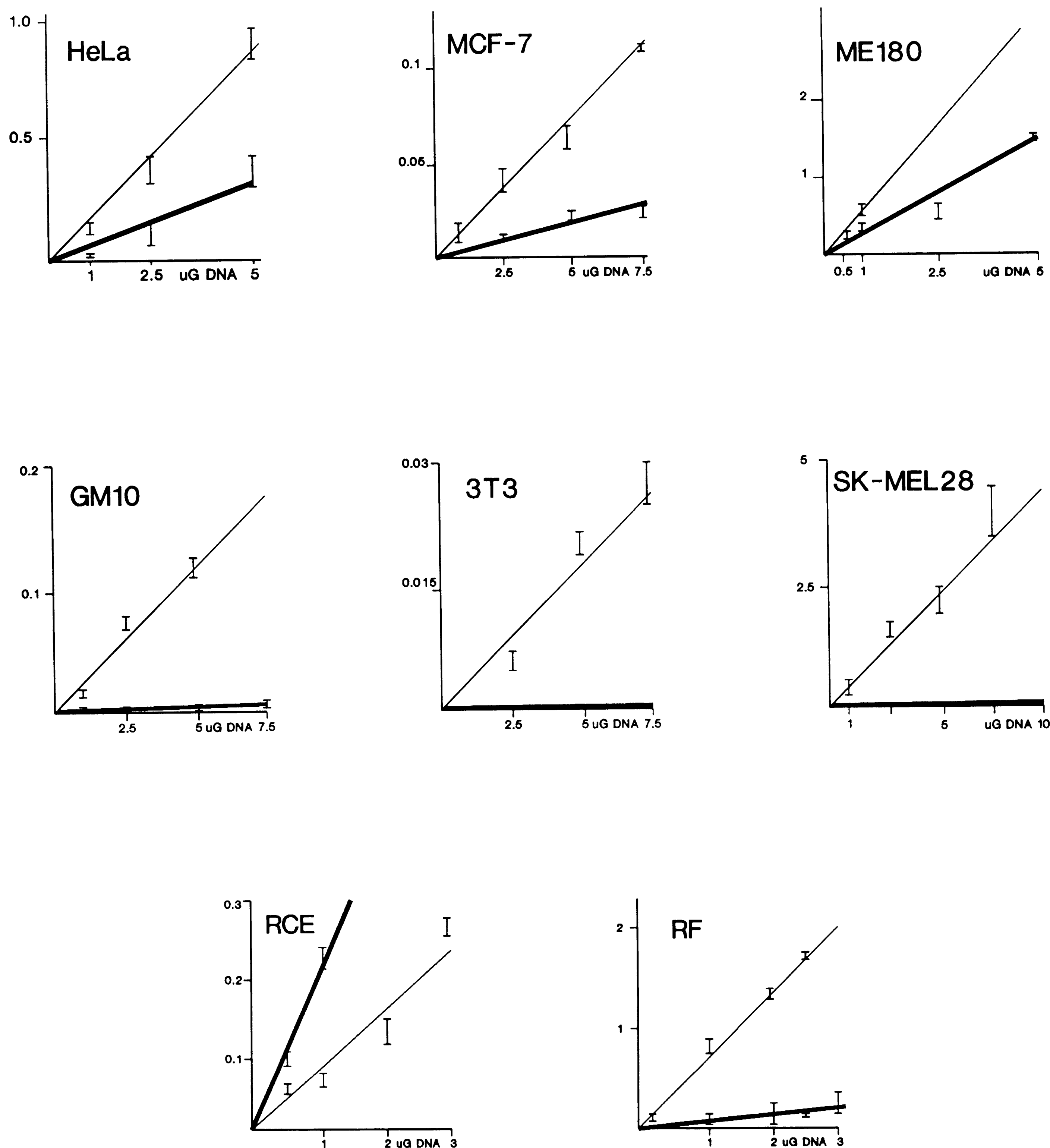

Figure 3: Normalized CAT activities are linear relative to the amounts of transformed DNA. The top row shows the data for epithelial cell lines, the middle for the non-epithelial cell lines and the bottom row for the primary cultures of rabbit corneal epithelial cells, RCF, and fibroblasts RF. The light lines represent the activities of pSV2-CAT, the heavy lines those of pK14-CAT. The CAT activities of the promoterless control plasmid pUC-CAT were virtually undetectable in all cell lines examined. Note that in the corneal cells, the activity of pK14-CAT is higher than the activity of pSV2-CAT.

than the viral promoter (Table I). In fibroblasts activity of the promoter, although detectable, was low. The epithelial specificity of the K # 14 keratin promoter was, therefore, evident in primary cultures as well.

We were concerned that, for some of the cell types used, the cells could be saturated with DNA and consequently yield misleading results. Therefore, we transformed each of the cell types with at least three different DNA concentrations. As shown

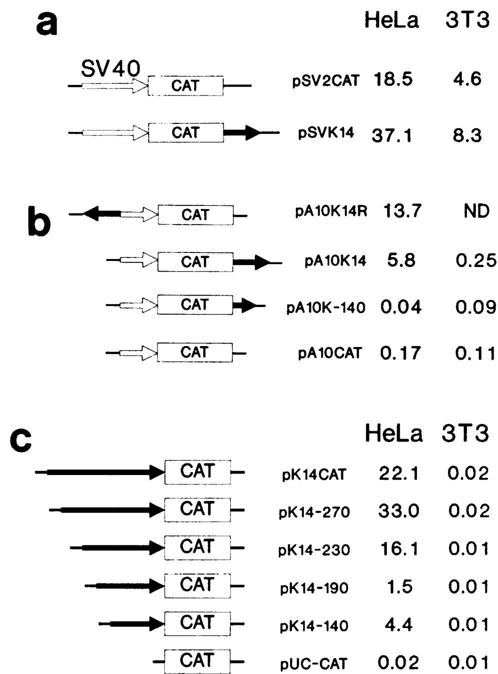

Figure 4a: The segment is not a silencer. The open arrow represents the SV40 regulatory region. Thin lines represent vector DNA, filled arrows the promoter DNA, the box the CAT gene. Normalized CAT activities for each plasmid in HeLa and 3T3 cells are shown on the right.
Figure 4b: The segment is an enhancer. Open arrow represents a weak enhancer-less promoter. ND, not done.
Figure 4c: Deletions of the promoter region. For exact 5′ ends of deletions consult Fig. 1.

in Figure 3, the CAT activities were linear relative to the amount of transfected DNA for all cell types, which means that the differences in CAT activities are not a result of variations in the DNA saturation levels but reflect different functional levels of the promoter.

The numbers in Table I have been normalized based on the internal control, i.e. the pRSVZ-derived B-GAL activities. pRSVZ activity is very high in 3T3 fibroblasts, but relatively low in ME180 and HeLa cells. The scales of the ordinates in Fig 3 were adjusted to compensate for these variations. The differences in the efficiency of the regulatory region of RSV-LTR were independently verified by transfection of the pRSV-CAT construct into 3T3 and ME-180 cells (data not shown).

Several mechanisms could account for the specificity conferred by the 300 bp segment. The two most obvious are a positive regulatory mechanism with an epithelial-specific activator, or a negative regulatory mechanism, implying an attenuator in all non-epithelial cells,. To test the second possibility, we have inserted the 300 bp segment downstream from the CAT gene of pSV2-CAT. If the segment had a strong, dominant silencing effect in non-epithelial cells, we would expect it to abate transcription from the SV40 promoter. On the contrary, as shown in Fig. 4a, the segment had a slight, two fold, positive effect on transcription from the viral promoter both in HeLa and in 3T3 cells. The 300 bp segment is, therefore, not a silencer, and we conclude that the epithelial-specific expression of the K # 14 keratin gene is not due to a negative regulatory mechanism.

Two types of positive cis-acting DNA segments have been described: promoters, functional only when positioned immediately upstream from the transcribed region, and enhancers, which can act at a distance, regardless of position or orientation (19). The 300 bp segment clearly has a promoter function, as described above, but in view of the results of Blessing et al. (17) who found an enhancer upstream from the bovine K # 6 keratin gene, we examined the enhancing capabilities of the 300 bp segment. We linked the segment to DNA containing the SV40 promoter which is completely dependent on a cis acting enhancer for expression of CAT (26). The 300 bp K # 14 gene segment does act as an enhancer since it can enhance transcription from the SV40 promoter, Fig 4b. The enhancement is not dependent on the orientation of the 300 bp segment and it is augmented in the construct containing multiple copies of the segment (data not shown). We conclude, therefore, that the 300 bp segment can function both as an enhancer and as a promoter.

The sequence of the 300 bp segment presented in Fig. 1 includes several consensus sites for various trans-activating proteins (91), as well as two segments similar to the epidermis-specific consensus, ESC, sequences (30). To determine which parts of the 300 bp segments are necessary for epithelial-specific expression of the K # 14 gene, we have constructed a series of deletions of the segment. Using the same reverse-oriented primer, Rev in Fig. 1, in conjunction with four different forward-oriented primers, we obtained four different DNAs by the polymerase chain reaction and cloned them in front of the CAT gene.

These constructs were introduced into HeLa and 3T3 cells (Fig. 4c). While the plasmids pK14-270 and pK14-230 were fully functional in epithelial cells, plasmids pK14-190 and pK14-160 were not, which identifies essential functional elements in the DNA between positions −230 and −190. These elements may be necessary for enhancer function since a construct containing the DNA between positions −1 and −140, but lacking the sequences further upstream was inactive in a pA10CAT background (Fig. 4b).

DISCUSSION

The immediate upstream region of the human K # 14 keratin gene is sufficient for epithelia specific gene expression. In this region we can identify, within 300 bp, two ESC sites at positions −240 and −130 (30), two CACCC sequences at −230 and −140 (31), two Sp1 consensus binding sites at −255 and −105, an AP1 site at −270 (29), and a TATA box at −90 (Fig 1). A smaller segment, missing the AP1 site and one each of the Sp1, ESC and CACCC sites, seems fully functional a promoter. The 40 bp sequence present in pK14-230 but not in pK14-190, in which we did not find any of the sequences reported necessary for gene regulation (29), is important for the high level of transcription of pK14-230.

What could be the reasons for the difference between, on one hand, our results and those of Blessing et al. (17) and, on the other hand, those of Vassar et al. (16) who, using a construct that contains 2.5 kb of the K # 14 gene upstream sequences, found promiscuous expression in fibroblasts? Formally, their disagreement with Blessing et al. could derive from using different keratin genes, K # 14 vs K # 6, or different organisms, man vs. cow, but we have also used a human K # 14 gene promoter, albeit one derived from a pseudogene (18). The fact that our sequence derives from a pseudogene is not the cause of the difference because this would require a pseudogene to gain, or maintain, a strict regulatory mechanism that the functional gene does not have. We believe that the discrepancy is due to the fact that Vassar et al. used a qualitative, not a quantitative, assay.

In a highly sensitive immunofluorescence assay, they scored as positive an occasional aberrant cell that expressed the transformed DNA at low levels. This was interpreted as promiscuous expression. We also find detectable levels of expression in primary cultures of rabbit fibroblasts (Table I), but our quantitative data show that the K # 14 keratin gene promoter is expressed up to several hundred times more in the epithelial cells than in the cells which do not normally express keratin intermediate filaments. We believe this is epithelial-specific regulation.

Patterns of keratin gene expression in various tissues are complex. In mammals, keratins are specific for epithelial cells (reviewed in 32). Simple epithelia express a different set of keratins than stratified epithelia (1). Within stratified epithelia, basal cells contain a specific set of keratins (6). Suprabasal cells can express hyperproliferation specific keratins (10), or keratins specific for various differentiation pathways. In the same organ, e.g. tongue or hair, adjacent cells can have different differentiation pathway-specific keratins (33−35). The acidic- and basic-type keratins assemble into filaments in exact stochiometric amounts. This stochiometry, maintained as the cell switches from expressing one set of keratins to another (9), is regulated both by synthesis and degradation (36,37).

Perhaps the simplest concept of keratin gene regulation is one in which the expression of each gene is regulated by several sets of *cis* and *trans*-acting factors that act as simple, binary, on-off switches. Such a model is consistent with our results: the 300 bp fragment confers epithelial specificity to K # 14 gene expression. The model is also consistent with the report of Blessing et al. who found a cell type specific enhancer in the upstream region of the bovine K # 6 gene (17). The same model can explain the regulation of expression of vimentin, a related, intermediate filament protein, since several investigators find that different DNA segments upstream from the gene have different effects on transcription in various cells (38−40).

Clearly, our 300 bp segment does not contain all the switches responsible for regulation of K # 14 keratin gene expression. Two of the cell types in Table I, HeLa and MCF-7, derive from simple epithelia and do not express their endogenous K # 14 gene (1,6). The expression of K14-CAT in HeLa cells was higher than in SCC-12 cells which normally synthesize keratin K # 14 (6). One possible explanation for this phenomenon could be that the endogenous K # 14 gene was permanently shut off in HeLa cells by DNA methylation, previously implicated in keratin gene regulation (41), at the time these cells became committed to simple epithelial, as opposed to stratified, differentiation. We have tested this hypothesis by digesting the DNAs from several cell lines with pairs of restriction enzymes differentially sensitive to methylation, Hpa II and Msp I, but we found that in all epithelial cell lines tested (although not in primary lymphocytes) the K # 14 promoter DNA was completely unmethylated (data not shown). The explanation we prefer for our data is that while the 300 base pair fragment contains information necessary for epithelial-specific expression of the K # 14 keratin gene, it does not contain the sites necessary for switching at the remaining levels.

Detailed analysis of the molecular mechanism by which the 300 bp segment confers epithelial specificity is under investigation, but we have already demonstrated position-independent, positive regulation. The position independence is illustrated in experiments that show that the segment has a strong enhancing capability (Fig. 4b), and that this enhancement is specific for epithelial cells (Fig. 4a).

In conclusion, we have discovered that the short, 300 base pair segment of DNA found in front of the K # 14 keratin gene is a functional promoter that confers epithelial-specific expression on the DNA downstream. While this segment may not contain all the cis-acting regulatory sites, it is recognized by the transcriptional machinery found in the nuclei of epithelial cells.

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