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A 300 bp 5′-upstream sequence of a differentiation-dependent rabbit K3 keratin gene can serve as a keratinocyte-specific promoter

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SUMMARY

Keratinocytes of the suprabasal compartment of many stratified epithelia synthesize as a major differentiation product a keratin pair, consisting of an acidic and a basic keratin, which accounts for 10-20% of the newly synthesized proteins. While genes of several differentiation-related keratins have been cloned and studied, relatively little is known about the molecular basis underlying their tissue-specific and differentiation-dependent expression. We have chosen to study, as a prototype of these genes, the gene of K3 keratin, which has the unique property of being expressed in the majority of corneal epithelial basal cells but suprabasally in peripheral cornea, the site of corneal epithelial stem cells. Using a monoclonal antibody, AE5, specific for K3 keratin, and a fragment of human K3 gene as probes, we have isolated several cDNA and genomic clones of rabbit K3 keratin. One genomic clone has been sequenced and characterized, and the identity of its coding sequence with that of cDNAs indicates that it corresponds to the single, functional rabbit K3 gene. Transfection assays showed that its 3.6 kb 5′-upstream sequence can drive a chloramphenicol acetyl transferase (CAT) reporter gene to express in cultured corneal and esophageal epithelial cells, but not in mesothelial and kidney epithelial cells or fibroblasts, all of rabbit origin. Serial deletion experiments narrowed this keratinocyte-specific promoter to within –300 bp upstream of the transcription initiation site. Its activity is not regulated by the coding or 3′-noncoding sequences that have been tested so far. This 300 bp 5′-upstream sequence of K3 keratin gene, which can function in vitro as a keratinocyte-specific promoter, contains two clusters of partially overlapping motifs, one with an NFkB consensus sequence and another with a GC box. The combinatorial effects of these multiple motifs and their cognate binding proteins may play an important role in regulating the expression of this tissue-restricted and differentiation-dependent keratin gene.

Key words: rabbit, K3 keratin gene, keratinocyte-specific promoter

INTRODUCTION

Cell-specific control of gene transcription plays a central role in the formation and maintenance of various tissue types, and is therefore a subject of major interest. A family of genes that is particularly suitable for such studies encodes about 30 keratin proteins, which form intermediate filaments in epithelial cells (Franke et al., 1981; Tseng et al., 1982; Moll et al., 1982; Heid et al., 1986; Lynch et al., 1986). These keratins can be divided into an acidic (type I) and a basic (type II) subfamily (Fuchs et al., 1981; Schiller et al., 1982; Tseng et al., 1982; Sun et al., 1984, 1985; Heid et al., 1986; Lynch et al., 1986). By studying the changes in keratin patterns as a function of tissue variation, development, diseased or cell culture conditions, and stages of stratified squamous epithelial differentiation, we noted that many specific ‘pairs’ of acidic and basic keratins tend to co-express in a tissue-restricted, developmentally-related, and differentiation-dependent fashion (Eichner et al., 1984; Sun et al., 1984; for additional references see Sun et al., 1985; Quinlan et al., 1985; Cooper and Sun, 1986; Steinert and Roop, 1988; Galvin et al., 1989). According to their mode of expression and their association with progressively more complicated epithelial structures, these keratin pairs can be divided into several categories (Schermer et al., 1989): (1) keratins of the simple epithelia include the K8/K18 pair and several other small MW keratins; (2) keratins expressed by the basal cells of all stratified squamous epithelia, the K5/K14 pair; (3) keratins expressed by the suprabasal cells of all types of stratified epithelia that become hyperplastic due to diseased or wound conditions or tissue culture stimuli, the K6/K16 pair; and (4) keratins

expressed by suprabasal cells of normally differentiating stratified squamous epithelia. This last group of keratins includes the K1/K10 keratins of keratinized epidermis, the K3/K12 keratins of corneal epithelium, and the K4/K13 keratins of esophageal epithelium. These keratins are highly tissue-restricted, are synthesized as the major differentiation products (accounting for 10-20% of the total proteins) of the epithelia, are largely responsible, as the major cytoskeleton, for the physical stability and rigidity of the fully differentiated cells and, in terms of their synthesis, are subject to exquisite regulation by retinoids, growth factors, extracellular calcium and matrix. Therefore, molecular regulation of genes encoding such keratins provides a paradigm for studying the tissue-specificity, the differentiation stage-related control, the co-regulation (of members of a keratin pair), and certain signal transduction aspects of gene expression.

Of all these differentiation-dependent keratin molecules, we have chosen to study the expression of K3, a basic 64 kDa keratin synthesized as a major differentiation product in corneal epithelium. This keratin has two attractive features. First, it is expressed suprabasally in the limbal (peripheral) region of the corneal epithelium, but uniformly (basal layer included) in central corneal epithelium. This observation has led us to propose that corneal epithelial stem cells are preferentially located in the limbus (Schermer et al., 1986; for additional experimental support of this hypothesis see Cotsarelis et al., 1989; Tsai et al., 1990; Tseng et al 1990; Lavker et al., 1991). This result also suggests that, unlike other differentiation-related keratin pairs whose expression is predominantly suprabasal, K3 can express under certain in vivo condition in a major subpopulation of (central corneal) basal cells. This raises the questions of whether this unusual basal cell expression relates to specific changes in the basement membrane of central cornea, as some immunological data appear to suggest (Schermer et al., 1986; Kolega et al., 1989), and, if so, what is the molecular mechanism for such a control. The fact that all the K3-negative corneal epithelial stem cells are clustered in the limbal region also provides unique opportunities for studying the differential gene expression in stem cells versus non-stem basal cells. Second, K3 keratin synthesis can be studied easily in cell culture. A useful approach for studying gene regulation involves constructing expression vectors in which various portions of gene sequences are placed upstream from a reporter gene, and testing the promoter-driven expression of the reporter gene in various cultured cells (Gorman et al., 1982, 1983). A prerequisite of this approach is a cell culture system capable of expressing and regulating properly the gene in question. In this regard, cultured rabbit corneal epithelial cells are uniquely suitable for studying the regulation of both differentiation and hyperproliferation-related keratin genes. Unlike most submerge-cultured human and mouse keratinocytes, which express relatively poorly their differentiation-related keratins, cultured rabbit corneal epithelial cells progress from initially synthesizing only the basal K5/K14 keratins, later (during exponential growth phase) synthesizing additional hyperproliferation-related K6/K16 keratins, and finally (when cells reach confluency and become heavily stratified) switching to synthesizing mainly the differentiation-related K3/K12 keratins (Schermer et al., 1986, 1989). Several other cultured rabbit cell types including esophageal epithelial cells, kidney epithelial cells, mesothelial cells and fibroblasts do not synthesize K3. Therefore a panel of cultured cells, all of the same rabbit origin, is available, and is ideally suited for studying the tissue-specific expression and differential regulation of K3 keratin gene.

In the present work, we have begun to analyze the promoter region of a rabbit K3 keratin (RK3) gene, which we have cloned and sequenced in its entirety. We provide evidence that 300 bp of its 5′-flanking sequence is sufficient to direct the keratinocyte-specific expression of a chloramphenicol acetyl transferase (CAT) reporter gene. Unlike the 5′-flanking sequence of most keratin genes, which contain relatively few binding motifs for known transcription factors, the 5′-flanking sequence of RK3 gene was found to contain two clusters of partially overlapping binding motifs of several known transcription factors, including NFkB and SP1. The availability of a relatively short (300 bp) K3 gene promoter containing sufficient information for keratinocyte-specific expression in a well-defined transfection assay will greatly facilitate further studies on the molecular mechanisms underlying the tissue-restricted and differentiation-dependent expression of this keratin gene.

MATERIALS AND METHODS

Cultured cells

Rabbit corneal and esophageal epithelial cells were isolated from young (1 month old) female New Zealand White rabbits and grown in Dulbecco's Modified Eagle's Minimum Essential Medium (DMEM) containing 18% fetal calf serum in the presence of mitomycin-treated 3T3 feeder cells as described earlier (Rheinwald and Green, 1975; Doran et al., 1980; Schermer et al., 1986). Rabbit mesothelial cells were isolated and cultured in the presence of 3T3 feeder cells according to Wu et al. (1982). A permanent cell line of rabbit kidney epithelial cells, originally established by Beale et al. (1963) and confirmed to be keratin-positive (data not shown), was obtained from the American Type Culture Collection and was grown in DMEM containing 18% fetal calf serum. Rabbit esophageal fibroblasts were grown in DMEM containing 18% fetal calf serum.

Protein analyses

Epithelial tissues or cultured cells were extracted with a buffer containing 25 mM Tris-HCl (pH 7.4), 1% Triton X-100 and a mixture of protease inhibitors, and the water-insoluble cytoskeleton was then dissolved in 25 mM Tris-HCl (pH 7.4), 9 M urea. The electrophoretic separation and immunoblotting of keratins were as described (Eichner et al., 1984, 1986).

Preparation and analysis of RNA

Cultured rabbit corneal epithelial cells were washed with ice-cold phosphate-buffered saline (PBS), and dissolved in 4 M guanidinium isothiocyanate, 10 mM Tris-HCl (pH 7.4) and 7% β-mercaptoethanol to yield total RNA. Poly(A)$^+$ mRNAs were isolated by oligo(dT)-cellulose chromatography (Maniatis et al., 1982).

Hybrid selection was basically performed according to Cleveland et al. (1980). Messenger RNAs from cultured rabbit corneal epithelial cells were hybridized with linearized and filter-bound plasmids containing various cDNA inserts. The bound mRNAs

were released in boiling water, and in vitro translated using a reticulocyte lysate system. The [^{35}S]methionine-labeled translational products were mixed with corneal epithelial keratins, which served as carrier proteins, and analyzed by one- and two-dimensional immunoblotting followed by autoradiography.

In situ hybridization was done according to Leube et al. (1986) with minor modifications. A 200 nucleotide ^{32}P-labeled antisense RNA probe was transcribed from a linearized plasmid using T3 RNA polymerase. This probe corresponded to the 3′-cDNA sequence encoding the C-terminal sequence of K3 keratin plus some non-translated sequences.

Isolation of cDNA and genomic clones

Messenger RNAs were isolated from confluent cultures of rabbit corneal epithelial cells that are known to synthesize K3/K12 keratins as their major differentiation products, and were used to construct cDNA libraries in pUC119 and λgt11. Screening of the cDNA libraries was done according to standard procedures (Maniatis et al., 1982). Genomic clones of rabbit K3 gene were isolated from two independent libraries, an EMBL-3 library purchased from ClonTech. Co. (Palo Alto, CA), and a Charon 4A library originally prepared by Maniatis et al. (1978; obtained from the American Type Culture Collection). The probes used for screening these libraries were labeled to a specific activity of > 10^6 c.p.m./μg by nick-translation. The hybridization, plaque purification and preparation of plasmid were done by standard techniques (Maniatis et al., 1982; Ausubel et al., 1989; Sambrook et al., 1989). Appropriate restriction fragments of these clones were subcloned into either pGEM or M13, and were sequenced using the dideoxynucleotide termination method (Sanger et al., 1977).

Southern blot analysis

DNA was extracted from the liver of a New Zealand White rabbit. A 10 μg sample of DNA was digested with various restriction endonucleases. The fragments were separated by electrophoresis through a 0.8% agarose gel, transferred to nitrocellulose membrane, and incubated with ^{32}P-labeled B13 probe (0.5-1×10^6 c.p.m./ml) in 2× SSC solution for 16 h at 42°C as described (Southern, 1975). The blots were washed three times, briefly, with 2× SSC solutions followed by three washes (55°C for 20 min.) in 0.5× SSC and 0.5% SDS.

Primer extension analysis

An antisense oligonucleotide (5′-CGGATGTCTTGCACACTTGT-3′) corresponding to position 9-28 bp downstream from the translational initiation codon was 5′-labeled using [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase. This primer was hybridized with the poly(A)$^+$ mRNAs of corneal or esophageal epithelial cells, and 5′-extended using reverse transcriptase (Sambrook et al., 1989). The products were resolved on a 6% polyacrylamide sequencing gel.

Construction of plasmids for transfection experiments

Tissue-specificity experiments (Table 1)

To construct an expression vector in which the CAT gene is driven by a 3.6 kb 5′-upstream sequence of RK3 gene (abbreviated pCAT3.6), we (i) digested RK3-G1 (a lambda genomic clone; Fig. 5) with *Eco*RI to isolate a 3.5 kb *Eco*RI-*Eco*RI fragment, (ii) synthesized a 160 bp PCR (polymerase chain reaction) fragment extending from the second *Eco*RI site (−87 bp from the transcription initiation site) to +76 (one base upstream from the translation initiation site), and (iii) ligated the above two fragments and inserted the product into the polylinker site of a pGCAT containing a bacterial chloramphenicol acetyl transferase gene (Hawley-Nelson et al., 1988). The pCAT1.5 and pCAT1.1 were similarly constructed using a 1.3 kb *Hin*dIII-*Eco*RI fragment and a 0.9 kb *Xba*I-*Eco*RI fragment, respectively, both ligated with the 160 bp PCR product. The pCAT0.4 was constructed using a PCR fragment extending from −348 to +76 (sequence shown in Fig. 8). All these constructs were confirmed by sequencing.

Serial deletion experiments (Fig. 9)

The pCAT 1.1 was digested with *Sph*I and *Sal*I to expose the 5′ end of the insert. Exonuclease III was added and incubated for increasing time periods to generate progressively shorter 5′-upstream deleted mutants. The precise lengths of these mutants were determined by sequencing.

Enhancer tests (Table 2)

The pCAT0.4 was digested with *Xba*I and Kpn I to release the 0.4 kb fragment extending from −348 bp to +76 bp (in relation to transcription initiation site; see Fig. 8), which was then blunt-ended inserted into the polylinker site of pCAT-basic (Promega; Madison, WI). Gene fragments, whose enhancer function was to be tested (E1 to E5, see Table 2), were then inserted into the *Bam*HI site located 3′-downstream from the CAT reporter gene.

Cell transfection

All plasmid DNAs were purified by two CsCl-ethidium bromide equilibrium centrifugations. Appropriate amounts of these expression vectors were used to transfect various cultured cells (60% confluent) using the calcium phosphate technique, basically according to Gorman et al. (1982). As a control for the transfection efficiency, a pRSVZ plasmid was used to co-transfect the cells so that the CAT activities could be normalized against the β-galactosidase activities in the same samples. The cells were harvested 40 h post-transfection when they reached a confluent and stratified state (in which all the suprabasal cells are already K3-positive). The amounts of the plasmids used for each experiments were titrated so that the observed CAT and β-galactosidase activities were both in the linear range. Typically, each 60 mm dish received 1 μg of CAT plasmid, 0.2 μg of pRSVZ plasmid and 3.8 μg of pUC119 (carrier plasmid DNA). The CAT activities of different constructs were calculated based on the molarity of the DNA inserts. The average of duplicates or triplicates, which usually showed less than 30% variation, was used.

RESULTS

Identification of rabbit K3 keratin cDNAs

To utilize the cultured rabbit corneal epithelial cells as a model system for studying keratin regulation, ideally we should use the K3 cDNAs and gene of the same species. Therefore we screened a λgt11 cDNA library of rabbit corneal epithelium (which expresses only two basic keratins, K3 and K5) with AE5 monoclonal antibody, which recognizes K3, as well as the 3′-sequence of a human ‘65 kDa keratin′ gene (Klinge et al., 1987), which we identified as a human K3 gene. Restriction mapping and nucleotide sequencing of the isolated cDNA clones indicated that they represented overlapping clones correspond to the 3′-half of a mRNA encoding a basic keratin. Several pieces of evidence showed that these cDNAs encoded K3 keratin. First, hybrid-selection using a short 3′-end cDNA (B13), corresponding to the C-terminal amino acid sequence which was keratin-chain-specific, resulted in the

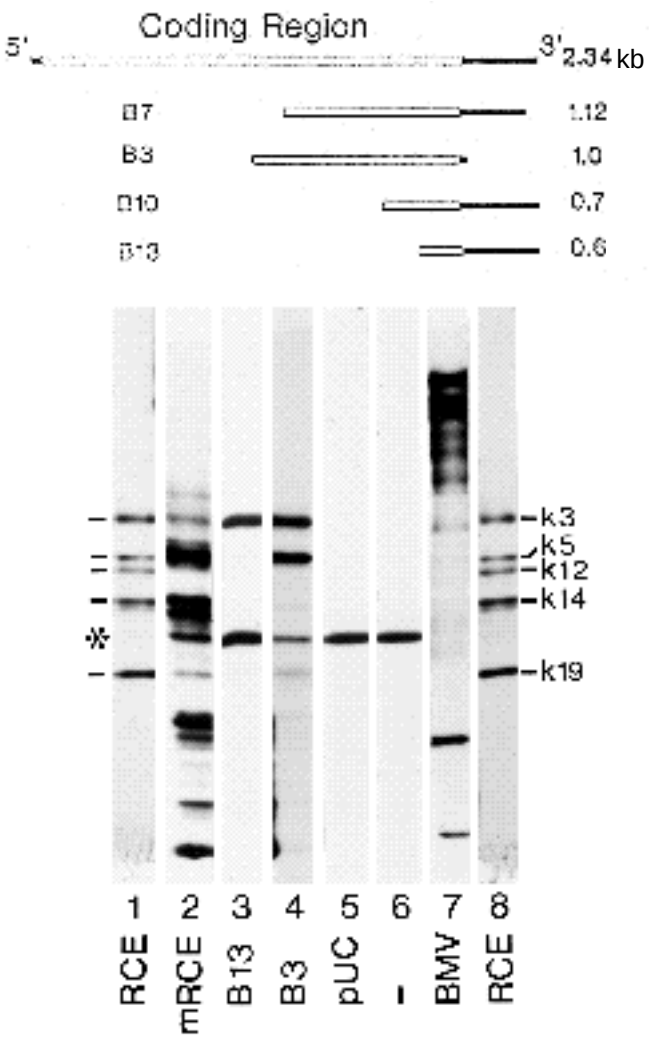

Fig. 1. Identification of rabbit K3 cDNA clones by hybrid selection. Total RNAs of a confluent culture of rabbit corneal epithelial cells were hybridized with linearized, filter-immobilized cDNA clones (B13 and B3). The hybrid-selected mRNAs were then eluted, in vitro translated in the presence of [^{35}S]methionine, and the products analyzed by SDS-PAGE. Samples are: lanes 1 and 8 controls showing the [^{35}S]methionine-labeled total keratins synthesized by a confluent culture of rabbit corneal epithelial cells; 2 in vitro translation products of total mRNAs of such a culture; 3 hybrid-selected products of B13 cDNA; 4 hybrid-selected products of B3 cDNA; 5 a control showing the hybrid-selected products of pUC plasmid containing no insert; 6 another control showing hybrid-selected products of filter paper alone; 7 a control showing the translation products of BM virus mRNAs. Note the hybrid-selection of K3 mRNA by the short 0.6 kb B13 cDNA clone which corresponds to the keratin-chain-specific and 3′-noncoding region of the K3 sequence. Also note that the longer B3 cDNA clone (1.0 kb), which encodes mainly the helical domain sequences conserved among basic keratins, hybrid-selected another basic keratin, K5, in addition to K3. Asterisk marks an artifact band present in all in vitro translated samples. The relationships among B3, B7, B10 and B13 cDNA clones and a theoretical full-length cDNA deduced from genomic clone (see later) are shown schematically above (the open and filled bars represent the coding and non-coding sequences, respectively).

selection of a mRNA that was translated into a single 64 kDa keratin, which co-electrophoresized with authentic rabbit K3 keratin during both one- and two-dimensional polyacrylamide gel electrophoreses (Figs 1 and 2). Second, the B13 probe recognized a 2.4 kb mRNA which was cornea-specific and differentiation-dependent, as it appeared only in confluent cultures of rabbit corneal epithelial cells, but not in esophageal epithelial cells (Fig. 3). Third, in situ hybridization using a 200 nucleotide antisense RNA probe (containing a part of B13 sequence) showed the presence of this messenger RNA in corneal epithelium but not in esophageal or skin epithelia or in any mesenchymal cells (data not shown). Fourth, sequencing data revealed that the C-terminal amino acid sequence of the keratin encoded by these cDNAs was SVKFSQSSQRYSR-COOH, which was highly similar to those of the human K3 (FSQSSQSSQRYSR-COOH) and bovine K3 (SVRF-SQSSQRTSR-COOH), but distinct from those of other known basic keratins (Jorcano et al., 1984; Klinge et al., 1987; Wu et al., unpublished). Together, these results proved unambiguously that our cDNA clones encoded rabbit K3 keratin.

Identification and characterization of a rabbit K3 gene

Although these cDNA clones covered only the 3′-half of the K3 keratin sequence, they provided adequate probes for RNA analysis and gene isolation. Southern blot analyses revealed that cleavage of rabbit genomic DNA with several restriction enzymes (*Bam*HI, *Bgl*II, *Hin*dIII, *Eco*RI, *Xba*I, *Xho*I) yielded a single DNA fragment that hybridized with the B13 probe (Fig. 4 and data not shown). Multiple, small B13-reactive restriction fragments were produced by *Pst*I (Fig. 4; lane 5); but this could be accounted for by the known existence of such restriction sites in K3 cDNA sequence. These results strongly suggested that K3 keratin was encoded by a single copy gene. To isolate this gene, we screened a Charon 4A and an EMBL-3 rabbit genomic library using the K3-specific B13 cDNA as a probe. Several partially overlapping clones (RK3-G1 to G4) were isolated (Fig. 5). The restriction map of these four genomic clones, as shown in Fig. 5, was in complete agreement with that obtained from the Southern blot analysis of rabbit liver genomic DNA (Fig. 4 and data not shown). The longest genomic clone, RK3-G1, was then characterized in detail. We sequenced approximately 6.5 kb of this clone, covering all the exons and introns as well as some of the 5′- and 3′-flanking sequences (Fig. 6). The exon-intron junctions of exons 5-9 were determined by a comparison of the genomic and available cDNA sequences. Most of these junctions matched the consensus sequences of 5′ and 3′-splicing sites. Significantly, the nucleotide sequences of the cDNA and genomic clones were found to be identical; this, plus the single copy nature of the gene (Fig. 4), strongly suggested that we had isolated the functional K3 gene. Since our partial cDNA clones did not cover exons 1-4, the exon-intron junctions of this area were tentatively assigned based on a combination of consensus splicing sequences, open reading frames, and the known junctional structures of several other Type II keratins (Johnson et al., 1985; Tyner et al., 1985;

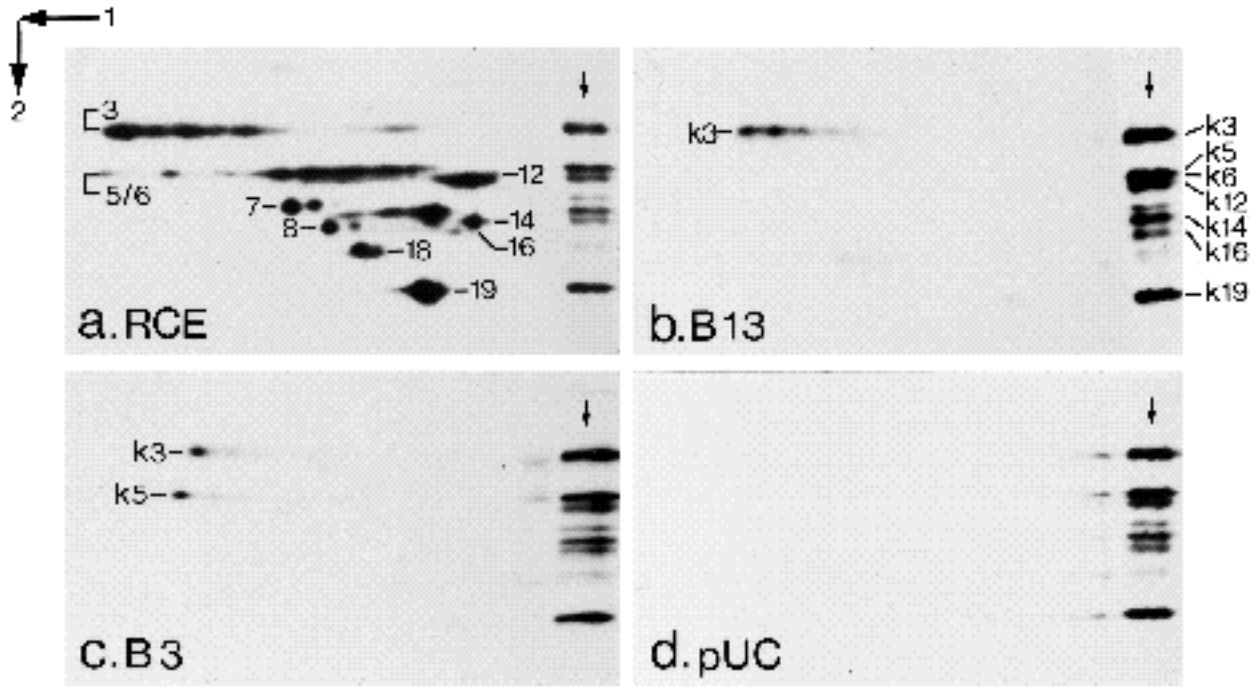

Fig. 2. Analysis of the hybrid-selected products by two-dimensional polyacrylamide gel electrophoresis. Hybrid-selected products of various cDNA clones, as described in Fig. 1, were mixed with unlabeled keratins isolated from a near confluent culture of rabbit corneal epithelial cells, separated by two-dimensional PAGE, and transferred to a nitrocellulose sheet. Following the visualization of the unlabeled carrier keratins by fast green staining (shown in a), the transfers were exposed to X-ray films to detect the hybrid-selected products of cDNA clone B13 (b), clone B3 (c), and pUC119 control (d). Arrows 1 and 2 denote the first dimensional separation by nonequilibrium pH gradient electrophoresis and the second dimensional separation by SDS-PAGE, respectively. The downward arrow marks a side lane resolving the total keratins of cultured rabbit corneal epithelial cells. Note the hybrid-selection of a single K3 keratin by B13 clone, and that of K3 plus K5 keratins by B3 clone.

Lersch et al., 1989; Krause and Franke, 1990; Tamai et al., 1991). The data thus indicated that the coding region of rabbit K3 gene contained 9 exons spanning about 6.0 kb (Fig. 5). This gene encoded a keratin with a theoretical molecular weight of 64,341 Da and an isoelectric point of 7.8; both matched extremely well with the known properties of rabbit K3 keratin (Figs 1 and 2). Its primary structure conformed to the general structural plan of type II keratins, with a central helical domain that could be divided into several subdomains (1A, 1B, 2A, 2B) with short non-helical interruptions (Steinert and Roop, 1988). The N- and C-terminal amino acid sequences of rabbit K3 keratin were glycine/serine-rich, and were capable of forming the so-called 'glycine loops' through the interactions among the intervening tyrosine/phenylalanine residues (Korge et al., 1992). Although this type of structure was first noted in K1 and K10 keratins of the epidermis, its presence in a differentiation-related keratin of non-keratinized corneal epithelium indicated that it was not unique to keratinization-products.

Transcription initiation site and 5′-upstream sequence

The transcription initiation site was determined by primer extension (Fig. 7). A major initiation site, located 30 bp downstream from a TATA box and 76 bp upstream from a presumptive translation initiation codon ATG, was identified (Fig. 7). Since a polyadenylation signal (AATAAA) was located 320 bp downstream from the translation stop codon, the predicted length of K3 mRNA is 2.315 kb (not including the poly A tail; Fig. 6), which was in close agreement with the actual size of approximately 2.4 kb (Fig. 3).

Within the 350 bp located upstream from the transcription initiation site, there were binding motifs of several known eukaryotic transcription factors (Fig. 8). One of these potential binding sequences was GGGGCTTTCC (located between −253 and −262 bp) which could bind NFkB, a rel-related protein known to play a key role in regulating the expression of some of the immunoglobulin genes during lymphocyte differentiation (Lenardo and Baltimore, 1989). Another sequence was CCCGCCCC (−197 and −204 bp) which could bind SP1, a zinc finger protein that could self-associate, bringing together distant DNA segments (Mastrangelo et al., 1991). Immediately downstream from this SP1 site was a 10 bp palindromic 5′-CCCTGCAGGG (at −189 to −198 bp), which shared 8 out of 10 bases with the Ker-1 site GCCTGCAGGC shown by Leask et al. (1990, 1991) as an AP2 binding site in the promoter region of human K14 gene (also see Snap et al., 1990). Several other potential binding sequences were also present, including those of SV40 core enhancer, AP2, NF1, and CK-8 (see Discussion).

Identification of a keratinocyte-specific promoter

To determine whether the 5′-upstream sequence of the isolated rabbit K3 gene could confer tissue-specificity, we con-

structed an expression vector in which a 3.6 kb upstream sequence of K3 gene, with its own TATA box, was used to drive the expression of a bacterial chloramphenicol acetyl transferase (CAT) reporter gene. This vector was co-transfected with a control vector containing a RSV promoter-driven β-galactosidase gene into several cultured rabbit cell types at about 60% confluency. Forty hours later the cells were harvested. The CAT activities were measured, and were normalized against the β-galactosidase activities which served as a control for the relative efficiency of transfection. Also as a control, we titrated the vector concentration-dependence of CAT activities and chose a vector concentration within a linear range (Evans and Scarpulla, 1989). The results as shown in Table 1, column 1, indicated that the 3.6 kb 5′-upstream sequence of rabbit K3 gene could drive CAT expression in cultured corneal and esophageal epithelial cells, but not in simple epithelial cells such as kidney epithelial cells or mesothelial cells, nor in fibroblasts. These results suggested that the tested K3 pro-

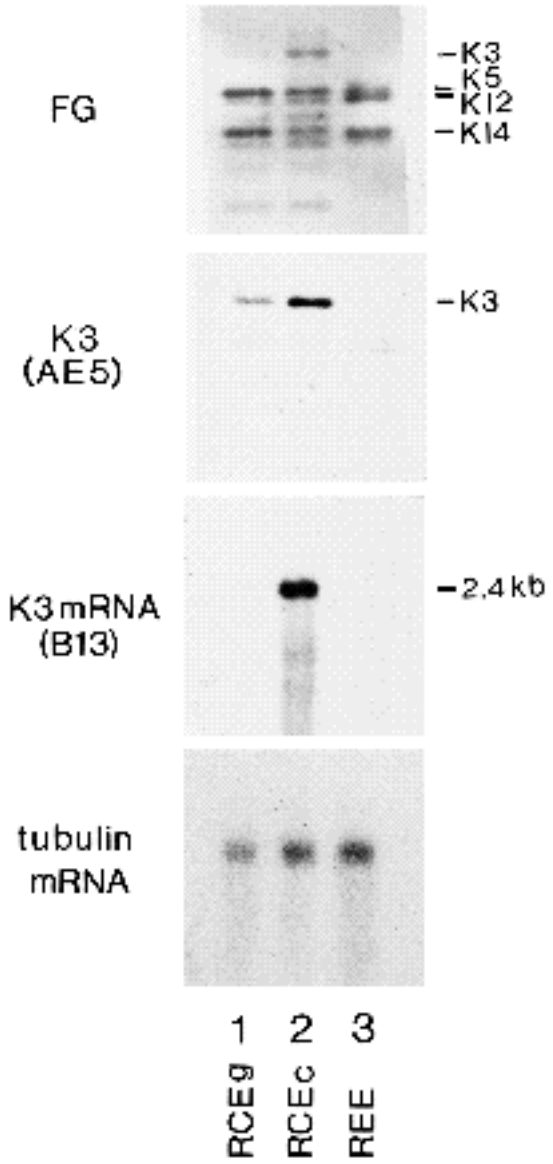

Fig. 3. The K3-specific B13 cDNA detects an mRNA that is corneal epithelium-specific and differentiation-dependent. Proteins of subconfluent (exponentially growing) rabbit corneal epithelial cells (lanes 1), confluent (and heavily stratified and differentiating) rabbit corneal epithelial cells (lanes 2), and confluent rabbit esophageal epithelial cells (lanes 3) were separated by SDS-PAGE, transferred electrophoretically to nitrocellulose sheet, and stained with fast green (FG; top panel), or with AE5 antibody which recognizes K3 keratin (second panel). Note the increase in K3 keratin protein (a marker for an advanced stage of corneal epithelial differentiation) in confluent corneal epithelial cells. Messenger RNAs were isolated from parallel cultures, separated by agarose gel, and hybridized with ^{32}P-labeled (K3-specific) B13 cDNA probe (third panel) or with a ^{32}P-labeled tubulin cDNA probe (bottom panel). Note the relative increase in K3 mRNA in confluent corneal epithelial cells, suggesting a regulation on the mRNA level. Also note the absence of K3 mRNA in esophageal epithelial cells.

Fig. 4. Southern blot analysis of rabbit K3 gene. A 10 μg sample of rabbit liver DNA was digested with saturating amounts of *Bam*HI (lane 1), *Bgl*II (2), *Hin*dIII (3), *Eco*RI (4) and *Pst*I (5). The DNA fragments were resolved by agarose (0.8%) gel electrophoresis, transferred to nitrocellulose paper, and hybridized with ^{32}P-labeled B13 cDNA probe specific for K3 keratin sequence. Note the generation of a single B13-positive fragment after *Bam*HI, *Bgl*II, *Hin*dIII and *Eco*RI digestions. The small fragments generated by *Pst*I (0.9 kb and 0.7 kb) can be explained by the presence of the restriction site within the B13 sequence. Size markers (kb) are shown on the right.

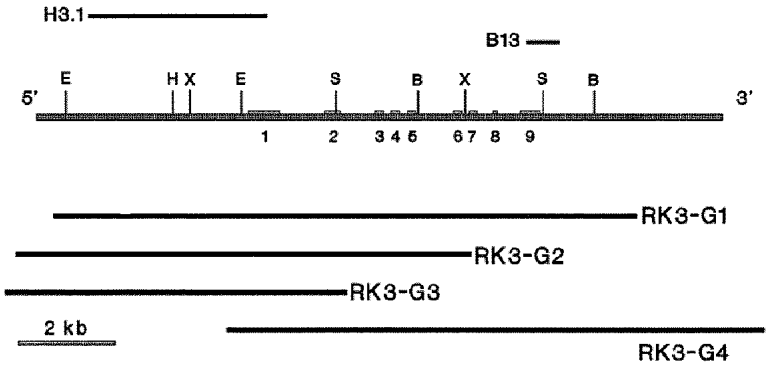

Fig. 5. A restriction map of rabbit K3 gene. A rabbit genomic library was screened with two probes: H3.1 (a 3.1 kb 5′-portion of human K3 gene), and B13 (a 0.6 kb rabbit K3 cDNA). Four genomic clones, G1, G2, G3 and G4, were obtained. Their relationships are as indicated, based on their restriction maps and partial sequences. Thicker bars numbered 1 through 9 denote the nine exons. Restriction sites are E(*Eco*RI), H(*Hin*dIII), X(*Xba*I), S(*Sph*I), and B(*Bam*HI).

moter sequence contained a keratinocyte-specific element(s), which was, however, insufficient for corneal specificity. Whether the 3.6 kb sequence can confer corneal specificity in vivo is unknown and needs to be tested using the transgenic mouse approach (also see Discussion).

In order to locate more precisely the keratinocyte-specific element(s) within this 3.6 kb sequence, we constructed 3 additional CAT expression vectors containing only 1.5 kb, 1.1 kb, and 0.4 kb of the upstream sequences, and used them to repeat the transfection experiments (Table 1). Full promoter activity with the same keratinocyte-specificity was found to be retained by all three constructs including the shortest one containing only 0.4 kb 5′-upstream sequence, indicating that the keratinocyte-specific element(s) must reside within this 0.4 kb sequence. Moreover, the fact that this 400 bp fragment was still not functional in non-keratinocytes suggested that (i) the keratinocyte-specificity of the promoter could not be attributed to a tissue-specific silencer located between −3.6 kb and −0.4 kb, and (ii) the promoter activity of this −0.4 kb required a (set of) transcription factor(s), functional only in keratinocytes.

A potential pitfall of using relatively large restriction fragments for the mapping of regulatory elements was that one might overlook some of the opposing elements which could neutralize each other. For example, detailed analysis of the 5′-regulatory sequences of vimentin and embryonic skeletal myosin heavy chain genes revealed a complex interplay of positive and negative elements (Bouvagnet et al., 1987; Sax et al., 1988; Stover and Zehner, 1992). Therefore we generated a series of 5′-deleted mutants by exonuclease treatment and used them to construct CAT expression vectors (Fig. 9). Transfection of these vectors into cultured corneal epithelial cells showed clearly that all 5′-sequences > 280 bp had about the same promoter activities (Fig. 9), indicating that under our assay conditions the sequence located between −3.6 kb and −280 bp did not affect the transcription efficiency. The promoter activity of sequences shorter than −282 bp decreased drastically, however, such that −250 bp sequence had only 60% activity, while those shorter than −137 bp showed low CAT activities approaching the background activity of the TATA boxless promoter (Fig. 9). These results confirmed those shown in Table 1, and indicated that an element important for keratinocyte-specific expression must reside within the region of −137 and −280 bp upstream from the transcription initiation site. These data of course could not rule out the existence of additional, downstream elements located between −137 bp and the transcription initiation site.

An unexpected aspect of our results was that the 5′-upstream sequence that we tested here could drive CAT expression not only in corneal epithelial cells, but also in esophageal epithelial cells which did not normally express K3 keratin. One possibility was that some of the (coding or 3′-downstream) gene sequences served as a silencer to suppress the promoter activity in non-corneal keratinocytes. To test this possibility, we divided the remaining sequence into five segments (E1 to E5) and inserted them, in both orientations, at the 3′-end of CAT gene that was driven by the 0.4 kb K3 promoter (Table 2). None of these inserts produced a significant change in the promoter activity and the keratinocyte-specificity of the −0.4 kb sequence (see below).

DISCUSSION

A keratinocyte-specific promoter that can be analyzed by transfection assay

In this paper, we have shown that a DNA segment containing 3.6 kb of 5′-upstream sequence of rabbit K3 gene can drive a CAT reporter gene to express efficiently in cultured rabbit corneal and esophageal epithelial cells (Table 1; Fig. 9) as well as rabbit epidermal cells (data not shown). However, this sequence is inactive as a promoter in simple epithelial cells such as rabbit mesothelial cells and kidney epithelial cells, nor in cultured rabbit fibroblasts (Table 1). That not all the 3.6 kb sequence is needed for such a keratinocyte-specific promoter function is shown by deletion experiments which indicate that fragments containing 1.5 kb, 1.1 kb, and 0.4 kb of 5′-upstream sequences apparently retain 100% of the promoter activity in cultured keratinocytes, and that even the smallest fragment containing only a 0.4 kb sequence still exhibits keratinocyte-specificity (Table 1). Since the promoter activity and tissue-specificity of this 0.4 kb fragment was not affected by any of the coding and 3′-noncoding sequences that we have tested so far (Table 2), this sequence must contain sufficient information for it to be able to function independently as a ker-

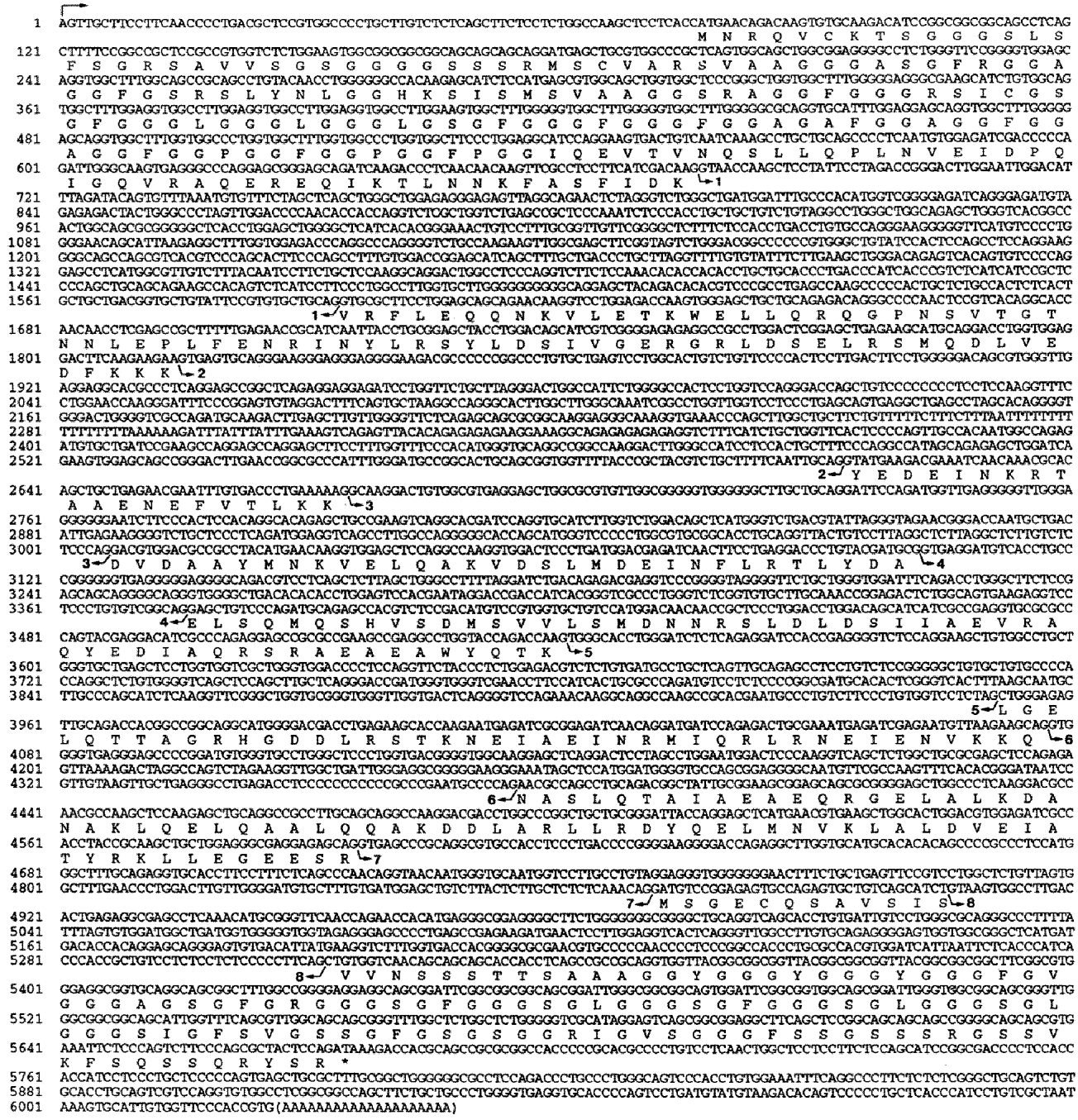

Fig. 6. The nucleotide sequence of rabbit K3 gene starting from its transcription initiation site to polyadenylation site, along with its deduced amino acid sequence. The exon/intron junctions (arrows with intron numbers) were determined by comparing the corresponding cDNA and genomic sequences and by a comparison with other known basic keratin genes (see text). The exons were theoretically translated into corresponding amino acids (one letter code). The transcription initiation site (designated as position 1) is marked with a horizontal arrow.

atinocyte-specific promoter. This conclusion is strongly supported by the transfection results shown in Fig. 9, in which a detailed and systematic deletion experiment revealed no significant regulatory elements between −3.6 kb and −280 bp. Although we do not yet know the mechanism by which this 280 bp 5′-sequence functions, the availability of this relatively short and well-characterized tissue-specific promoter that can function in transfection assays can facilitate detailed studies on the roles of various motifs and their cognate binding proteins in controlling the keratinocyte-specific expression of K3 gene.

Importance of analyzing promoter function in cells of the same species

Although a panel of cells from several species has frequently been used in transfection assays to study the tissue-

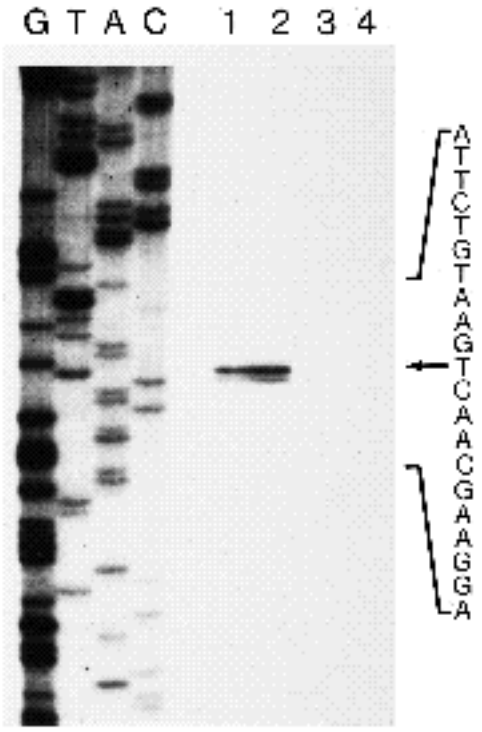

Fig. 7. Identification of the transcription initiation site of rabbit K3 gene. An antisense oligodeoxynucleotide (20mer) corresponding to a sequence starting 9 bp downstream from the translation-initiation site was synthesized and used in a primer extension assay. The templates were poly(A)$^+$ mRNA from a confluent culture of rabbit corneal epithelial cells (lane 1, 1 μg; lane 2, 2 μg), poly(A)$^+$ mRNAs from rabbit esophageal epithelial cells (lane 3, 1 μg), or yeast tRNA's (lane 4, 1 μg). Lanes labeled G, T, A, C are sequencing gels for comparison. Note the formation of a well-defined, corneal-specific primer extension product in lanes 1 and 2 corresponding to a transcriptional initiation site 76 bp 5′-upstream from the ATG translational initiation site.

specific expression of genes, we have decided to limit all our analyses to cells of rabbit origin. This is because even though some genes have been shown to behave indistinguishably in cells of foreign species, many examples exist in which genes exhibit striking species-specificity (Muscat and Kedes 1987; Grayson et al., 1988). In fact the importance of this consideration is underscored by our own recent observation that the 5′-upstream sequence of human K3 gene deviates significantly from that of the rabbit K3 gene, and that a much longer segment (>2.5 kb) of 5′-upstream sequence of human K3 gene is required for it to function as a keratinocyte-specific promoter (R.-L. Wu et al., unpublished).

Why is the 5′-upstream sequence of RK3 gene keratinocyte-specific but lacking corneal specificity?

Our data indicate that a 3.6 kb segment of 5′-upstream sequence of rabbit K3 gene can function as a promoter not only in corneal keratinocytes (which normally express K3 gene) but also in esophageal keratinocytes (normally do not express K3; Fig. 3). There are two possible explanations for this lack of corneal specificity. First, some regulatory motifs involved in defining corneal specificity may be missing in the 3.6 kb 5′-upstream region that we have analyzed so far. Alternatively, the 3.6 kb sequence, 'naked' and isolated from other chromosomal components, may contain all the necessary regulatory motifs but lacks certain modifications such as methylation, or lacks specific structural constraints imposed by local chromosomal structure, which normally shuts off K3 gene in esophageal or other K3-negative keratinocytes. Methylation and chromosomal structure have been shown to play an important role in suppressing the expression of at least K14 and K18 genes in certain 'negative' tissues, as evidenced by the fact that particular genomic sequences can sometimes exhibit tissue-specificity in transgenic mouse but are lacking such tissue-specificity in in vitro transfection assays (Kulesh and Oshima, 1988; Vassar et al., 1989; Abe and Oshima, 1990).

Promoters of relatively few keratin genes exhibit keratinocyte-specificity in transfection assays

Although the genes of many keratins have been cloned, the 5′-upstream sequences of only a few of them have been found to function as tissue-specific promoters in transfection assays. Blessing et al. (1989) showed that a 600 bp 5′-upstream sequence of a bovine K6 gene can drive a CAT reporter gene to express in a bovine mammary gland-derived, keratinocyte-like cell line and a mouse keratinocyte cell line (AT-5). This promoter appears to be inactive in a bovine mammary-derived simple epithelial cell line, bovine kidney epithelial cells, and mouse 3T3 fibroblasts, suggesting that it functions as a keratinocyte-specific promoter. Jiang et al. (1990, 1991) showed that short segments of 5′-upstream sequences of human K6b (390 bp) and K10 (1200 bp) can drive CAT expression in cultured rabbit corneal and esophageal epithelial cells, but not in rabbit mesothelial cells, human HeLa cells or mouse 3T3 fibroblasts. They also showed that a 560 bp 5′-upstream sequence of human K5 gene can function as a keratinocyte-specific promoter, although in more recent studies this promoter appears to function well in HeLa cells (Ohtsuki et al., 1992). Whether the 5′-upstream sequence of human K14 gene can function in vitro as a keratinocyte-specific promoter also appears to be complicated. Although a 2.3 kb 5′-upstream sequence was shown to be able to define keratinocyte-specific expression in transgenic mouse, this DNA sequence was first found to be able to function in an in vitro transfection assay in 3T3 fibroblasts (Vassar et al., 1989). Later, this upstream sequence was reported to be involved in controlling keratinocyte-specific expression in vivo and in vitro (Leask et al., 1990). More recent transfection experiments showed, however, that this 5′-upstream sequence can drive a CAT reporter gene to express in HeLa cells 4.8 times better than in keratinocytes, indicating that it cannot be keratinocyte-specific (Leask et al., 1991). In another independent study, Jiang et al. (1990) showed that a 390 bp 5′-upstream sequence of human K14 pseudo-gene can drive the expression of a CAT reporter gene to express in cultured rabbit keratinocytes as well as HeLa cells, but not in mouse 3T3 fibroblasts. Taking these data together, it now appears that the 5′-upstream sequence of K14 gene can function in transfection assays as an epithelial-specific, but

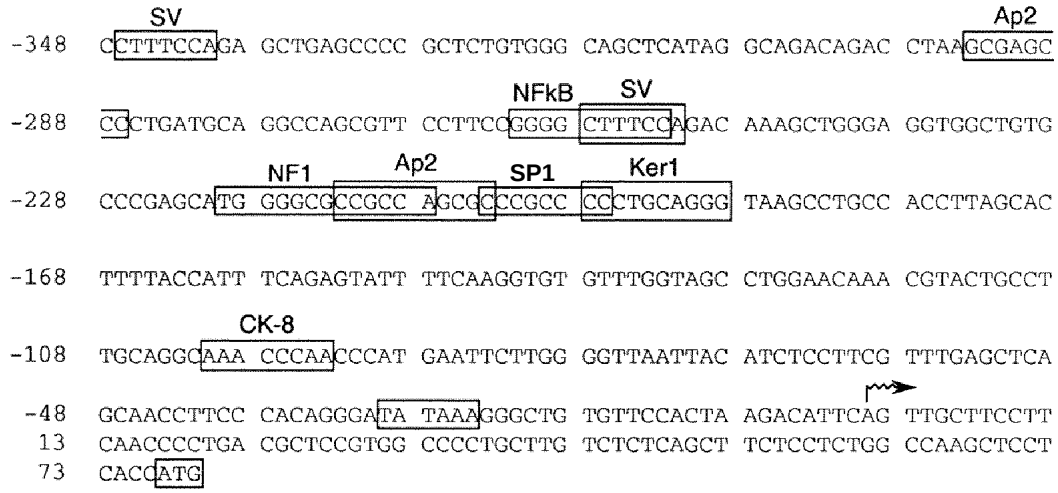

Fig. 8. Nucleotide sequence and some potential regulatory elements of the 5′-upstream region of rabbit K3 gene. The transcriptional initiation site, marked by a wavy arrow, is assigned as position 1, and the base 5′-upstream next to it, −1. Boxes indicate the potential binding sites of, from 5′-end, SV(SV40 core enhancer, −341 to −347; −253 to −258; Pfeifer et al., 1987), AP2 (−287 to −294; −204 to −213; Imagawa et al., 1987; Williams and Tjian, 1991), NFkB (−253 to −262; Collart et al., 1990), NF1 (−208 to −220; Leegwater et al., 1986), SP1 (−197 to −204; Ishii et al., 1986), Ker 1 (−189 to −198; Leask et al., 1990, 1991), CK-8 (−94 to −101; Blessing et al., 1987). The TATA box and translation initiation codon ATG are also boxed.

Table 1. Tissue-specificity of the rabbit K3 gene promoter

	Length of K3 promoter			
Rabbit cell type	3.6	1.5	1.1	0.4 kb
Corneal epithelium	19	21	25	27
Esophageal epithelium	17	13	19	14
Kidney epithelium	1	1	1	1
Mesothelium	<1	<1	<1	<1
Fibroblast	1	1	1	1

The promoter activity of various lengths of K3 upstream sequences, as measured by their ability to drive the expression of a bacterial chloramphenicol acetyl transferase gene, was assayed by transfecting the expression vectors into a panel of cultured rabbit cell types (for the construction and sequence confirmation of these vectors, see Materials and Methods). A co-transfected vector containing a β-galactosidase gene driven by a RSV promoter provided a control for transfection efficiency. For each cell type, the ratio of the CAT activities of various vectors over that of a promoter-less vector were calculated. Note the full activity and keratinocyte-specificity of even the shortest 0.4 kb K3 promoter.

not keratinocyte-specific, promoter. Finally, the 5′-upstream sequences of several other keratin genes including the simple epithelial keratin gene K18 do not seem to possess any tissue-specificity, as they are functional even when transfected into mesenchymal cells (Oshima et al., 1988, 1990).

Collectively, these data indicate that the 5′-upstream sequences of relatively few keratin genes including K6, K10 (possibly) and, as shown here, K3, can function in transfection assays as keratinocyte-specific promoters. This observation underscores the importance of distal elements and/or chromosomal structure in regulating the expression of keratin genes in general (Oshima et al., 1988; Leask et al., 1990). It also suggests that the few keratin genes, such as K3, whose promoters exhibit tissue-specificity in transfection assays can provide particularly convenient and important models for further studies on the molecular basis of the tissue-specific regulation of keratin genes.

An important feature of RK3 promoter: abundant and partially overlapping binding motifs of transcription factors

The three previously described keratinocyte-specific promoters (of bovine and human K6 gene, human K10 gene, and possibly human K5 gene) contain several binding motifs for known transcription factors. Thus the 600 bp of 5′-upstream sequence of bovine K6 gene is known to contain two AP1 and one AP2 binding site, and the 800 nucleotides of 5′-upstream of human K5 gene contains motifs related to the SV40 core enhancer (Eckert and Rorke, 1988), AP2 binding site CCCCAGGC (Lersch et al., 1989), and several other sites (Ohtsuki et al., 1992). These sites are in general well separated from one another. In contrast, the −300 bp of 5′-upstream sequence of rabbit K3 gene contains a large number of binding motifs for known transcription factors, and many of them partially overlap with each other forming two major clusters (Fig. 8). The cluster located more upstream (−250 to −262) contains: (i) A sequence 5′-GGGGCTTTCC-3′, located at −253 to −262, which fits perfectly the consensus binding sequence of NFkB (GGG A/G A/C T T/C T/C CC). Our preliminary data from gel retardation and supershift assays indicate that this motif indeed binds an NFkB-related protein (R.-L. Wu et al., unpublished). NFkB and other rel-related proteins have been shown earlier to be involved in the tissue-specific and differentiation-dependent expression of several ‘defense’ molecules including the κ-light chain of immunoglobulin, β-interferon, T-cell receptor, TNF-λ and TNF-β (Lenardo and Baltimore, 1989; Blank et al., 1992). (ii) A sequence 5′-CTTTCCAGA-3′, partially overlapping with the NFkB site, is located at −250 to −258 (also present as a direct repeat at −339 to −347). Sequences related to the SV40-core enhancer element 5′-CTTTCCA-3′ are present in several keratinocyte-specific keratins including human K6, K14 and K17. However, as Eckert and Rorke (1988) pointed out earlier, this kind of sequence is not

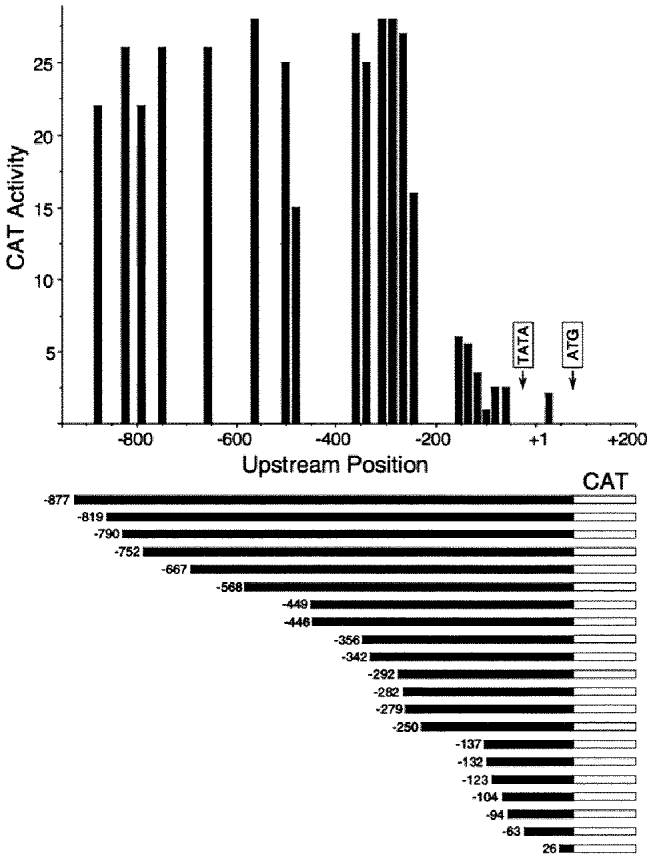

Fig. 9. Detection of a 5′-upstream sequence of K3 gene capable of driving a chloramphenicol acetyl transferase (CAT) reporter gene to express in cultured corneal epithelial cells. A series of expression vectors was constructed in which various lengths of K3 promoter were positioned upstream from the CAT reporter gene; the length and sequence of all these truncated promoters were confirmed by sequencing (see Materials and Methods). The vectors were transfected into cultured rabbit corneal epithelial cells, and 40 h later the cultures (stratified with K3-positive superbasal cells) were harvested and the CAT activities were measured. As a control for the transfection efficiency another expression vector (containing a β-galactosidase gene driven by the RSV promoter) was used to co-transfect the cells, and the β-galactosidase activities were used to normalize the CAT activities. The numbers at the *x*-axis and in the lower bar diagram denote the bp positions upstream from the transcription initiation site. Note that deletion of sequences into the area of −280 to −120 resulted in a drastic decrease in K3 promoter activity.

Table 2. Assay for a possible enhancer activity in rabbit K3 gene

Promoter	E		CAT activity RCE	CAT activity REE
SV40	0		2.7(±0.5)	2.17(±0.58)
NONE	0		<0.04	0.025
K3 (0.4 kb)	0		1.0(±0.09)	1.0(±0.14)
K3 (0.4 kb)	E1	→	0.9(±0.03)	1.01(±0.13)
	E1	←	1.0(±0.04)	1.05(±0.16)
	E2	→	0.8(±0.2)	1.27(±0.24)
	E2	←	0.9(±0.2)	1.25(±0.25)
	E3	→	0.7(±0.02)	0.97(±0.14)
	E3	←	0.8(±0.04)	1.12(±0.18)
	E4	→	1.4(±0.1)	0.89(±0.23)
	E5	←	1.0(±0.04)	1.04(±0.09)

The coding and a portion of the 3′-noncoding region of K3 gene, spanning a total of ~7.4 kb, was divided into 5 consecutive (5′→3′) restriction fragments designated E1-E5. They were E1 (extending from the second *Eco*RI site of K3 gene, as defined in Fig. 5, to the first *Sph*I site, 2 kb containing exon 1, intron 1 and a part of exon 2); E2 (S→B/1.72 kb/exons 2*, 3, 4, 5); E3 (B→X/1.04 kb/exon 6); E4 (X→S/1.6 kb/exons 7, 8, 9); and E5 (S→B/1.04 kb/3′-end). These restriction fragments were inserted, in both directions (→ means 5′→3′), downstream from a CAT gene driven by the 0.4 kb K3 promoter (for the construction and sequence confirmation of these vectors, see Materials and Methods). These vectors were transfected into cultured rabbit corneal epithelial (RCE) or esophageal epithelial (REE) cells, and 40 h later the CAT activities were measured and compared with the β-galactosidase activities (internal controls). In this experiment, the CAT activity of K3 (0.4 kb) vector was defined as 1.0 in both cell types. The numbers represent the average of three independent measurements followed by standard deviations (in parentheses). Note that there was no evidence for a tissue-specific silencing or enhancing elements.

present in, e.g. K5 promoter suggesting that it is not always required for keratinocyte-specific expression.

The second cluster (−189 to −220) contains four overlapping motifs: (a) located at −208 to −220 is a sequence 5′-TGGGGCGCCGCC-3′ that is similar to the consensus binding sequence (5′-TGG A/C A/T NNN A/T GCCAA-3′; Leegwater et al., 1986) of NF1, a 52-66 kDa nuclear factor inducible by TGF-β (Courtois et al., 1990). (b) Downstream and partially overlapping with the NF1 site is a potential AP2 site (−204 to −213) with a sequence of 5′-CCGCCAGCGC-3′ (consensus 5′-CCC A/C N G/C G/C G/C-3′; Faisst and Meyer, 1992). This 50 kDa AP2 protein is inducible by TPA, cAMP and retinoic acid (Williams et al., 1988). (c) Downstream and partially overlapping with the AP2 site is a binding site for SP1 (5′-CCCGCCCC-3′ at −197 to −204), which usually serves as a positive regulator (Schmidt et al., 1989; Jackson et al., 1990). We have obtained preliminary data from gel retardation, supershift and mutagenesis experiments indicating that an SP1-like nuclear protein of rabbit corneal epithelium indeed binds to this motif and that this binding is functionally important (R.-L. Wu et al., unpublished). (d) A sequence (5′-CCCT-GCAGGG-3′), located at −189 to −198, is similar to the Ker-1 site (5′-GCCTGCAGGC-3′) of human K14 gene. Although it was originally suggested that the cognate protein of this binding motif was involved in controlling the keratinocyte-specific expression of K14 gene (Leask et al., 1990), this DNA-binding protein was later found to be AP2 and was in itself insufficient for tissue-specific expression (Leask et al., 1991). However, the fact that in the keratinocyte-specific promoter of K3 gene this potential AP2 binding site partially overlaps with a GC box raises interesting possibilities of regulation.

Three other isolated motifs are present. At −94 to −101 a sequence, 5′-AAACCCAA-3′, was located which was similar to the CK-8 sequence of 5′-AANCCNAA-3′. Blessing et al. (1987) noted this sequence in the 5′-upstream region of several epidermally expressed genes. The fact that this motif is also present in K3 gene, which is strongly expressed in corneal, snout, and some of the oral mucosal epithelia, indicates that this motif may function in both epidermal and non-epidermal keratinocytes. Another potential

AP2 site is present at −287 to −294. Finally, a typical TATA box is located between −25 and −30.

In conclusion, we have established that a 280 bp 5′-upstream sequence of rabbit K3 keratin gene can function as a keratinocyte-specific promoter in transfection assays. This promoter region is unique among those of keratin genes that have been studied so far in that it contains two clusters of partially overlapping binding motifs for several transcriptional factors including NFkB and SP1, which, in conjunction with proteins binding to their neighboring and partially overlapping motifs, can potentially play a role in regulating the expression of RK3 gene. As we learn more about the functional significance of these binding motifs and their cognate transcription factors from ongoing gel retardation, mutagenesis and footprinting experiments, we hope to better understand the molecular basis underlying the tissue-restricted and differentiation-dependent expression of this important keratin gene.

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