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Isolation, Sequence, and Expression of a Human Keratin K5 Gene: Transcriptional Regulation of Keratins and Insights into Pairwise Control

ROBERT LERSCH, VERONICA STELLMACH, CLIFFORD STOCKS, GEORGE GIUDICE, AND ELAINE FUCHS*

Department of Molecular Genetics and Cell Biology, Howard Hughes Medical Institute, The University of Chicago, Chicago, Illinois 60637

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The mitotically active basal layers of most stratified squamous epithelia express 10 to 30% of their total protein as keratin. The two keratins specifically expressed in these cells are the type II keratin K5 (58 kilodaltons) and its corresponding partner, type I keratin K14 (50 kilodaltons), both of which are essential for the formation of 8-nm filaments. Dissecting the molecular mechanisms underlying the coordinate regulation of the two keratins is an important first step in understanding epidermal differentiation and in designing promoters that will enable delivery and expression of foreign gene products in stratified squamous epithelia, e.g., skin. Previously, we reported the sequence of the gene encoding human K14 (D. Marchuk, S. McCrohon, and E. Fuchs, Cell 39:491–498, 1984; Marchuk et al., Proc. Natl. Acad. Sci. USA 82:1609–1613, 1985). We have now isolated and characterized the gene encoding human K5. The sequence of the coding portion of this gene matched perfectly with that of a partial K5 cDNA sequence obtained from a cultured human epidermal library (R. Lersch and E. Fuchs, Mol. Cell. Biol. 8:486–493, 1988), and gene transfection studies indicated that the gene is functional. Nuclear runoff experiments demonstrated that the K5 and K14 genes were both transcribed at dramatically higher levels in cultured human epidermal cells than in fibroblasts, indicating that at least a part of the regulation of the expression of this keratin pair is at the transcriptional level. When the K5 gene was transfected transiently into NIH 3T3 fibroblasts, foreign expression of the gene caused the appearance of endogenous mouse K14 and the subsequent formation of a keratin filament array in the cells. In this case, transcriptional changes did not appear to be involved in the regulation, suggesting that there may be multiple control mechanisms underlying the pairwise expression of keratins.

Only epithelial cells express keratins, a large family of proteins (molecular size, 40 to 67 kilodaltons) (kDa) that assemble into a cytoskeletal network of 8-nm intermediate filaments (IFs). There are two distinct classes of keratins: type I keratins (K9 to K19) are small (40 to 56.5 kDa) and relatively acidic (pK_i, 4.5 to 5.5), whereas type II keratins (K1 to K8) are larger (53 to 67 kDa) and more basic (pK_i, 5.5 to 7.5) (for reviews, see references 8, 16, 50, and 64). Both types of keratins are essential for filament assembly (12, 28, 41, 63). Type I and type II keratins are frequently expressed as specific pairs, and different pairs are expressed in a tissue-specific, differentiation-specific, and developmental-specific fashion (11, 66).

The molecular mechanisms underlying the regulation of pairs of type I and type II keratins remain largely unknown. It is clear that self-propagating epithelial cells expressing a specific pair of keratins must necessarily cotranscribe these genes. However, coordinate expression does not restrict these genes to a common regulatory mechanism, nor does it rule out the possibility that the two genes may be differentially transcribed in other situations. In addition, posttranscriptional regulation may be involved in controlling the pairwise expression of keratins. That the pairwise regulation of keratins is complex is suggested by the observation that in the course of differentiation, type II keratins often appear before their type I partners (10, 36, 61). Moreover, gene transfection studies using nonepithelial cells have demonstrated the appearance of a type I keratin (unidentified) as a consequence of expression of a foreign type II keratin (K6) (19). Hence, at least to some extent, the expression of type I keratins may depend on expression of their type II partners.

The epidermis of the skin provides an important model for examining the molecular details of keratin gene expression. Keratins are especially abundant in this tissue, accounting for as much as 30% of the basal cell protein and 85% of the protein of fully differentiated squames. In the basal layer, two keratins are expressed: a type I keratin K14 and a type II partner keratin K5 (51). As a basal cell undergoes a commitment to terminally differentiate, it down regulates expression of this keratin pair and induces expression of new keratins of both the type I class (K10 and K11) and the type II class (K1 and K2) (14, 36, 72). In diseases of hyperproliferation and during wound healing of the skin, a new set of keratins, consisting of K6, K16, and K17, is induced (45, 65, 73). Although there is some evidence of translational control of the keratins expressed in skin (71), most of the changes are at the mRNA level (14, 43, 65, 71). No studies have yet been conducted to determine the extent to which keratin expression is regulated at the transcriptional level in keratinocytes.

Of the keratin pairs expressed in epidermis, we have chosen K5 and K14 for our studies, since this pair is expressed in the mitotically active basal cells of most stratified squamous epithelia (9, 35, 51, 65). Elucidating the mechanisms underlying the regulation of these keratins will be useful not only for our understanding of epidermal differentiation but also for constructing promoters to drive the expression of foreign genes in epidermis. Previously, we isolated, characterized, and sequenced the human K14 gene

* Corresponding author.

(46, 47). We now report the isolation, sequence, and characterization of the human K5 gene. In addition, we provide a comparison of the 5′ upstream region of the K5 gene with the corresponding region of its partner gene, and we use nuclear runoff transcription experiments to demonstrate transcriptional regulation in cells of stratified squamous epithelial and nonepithelial origin. Finally, we examine expression of the K5 gene introduced into NIH 3T3 cells. Our results provide new insights into the regulation of type II and type I keratin pairs.

MATERIALS AND METHODS

Screening of the genomic library. A human genomic library (*Hae*III-*Alu*I partial digest of human genomic DNA, cloned into the *Eco*RI site of lambda bacteriophage Charon 4A through the use of linker ligation), was obtained from Ed Fritsch (Genetics Institute, Boston, Mass.). Phage were screened essentially as described by Benton and Davis (2). For probes, two subclones were made from a previously sequenced K5 cDNA (KA-62; 43). A subclone corresponding to the 5′ end of the K5 cDNA was made by inserting a 377-base-pair (bp) fragment from KA-62 into the *Pst*I site of plasmid pGEM2 (Promega Biotec). This subclone, referred to as pK5-5′, encompasses 11 bp of 5′ poly(G) sequence (from the terminal transferase method of preparation of the cDNA library) and 366 bp of 5′ coding sequence. To make cRNA probes, pK5-5′ was linearized with *Hin*dIII and transcribed by using SP6 RNA polymerase. A subclone corresponding to the 3′ end of the K5 cDNA was made by inserting a 190-bp *Nco*I-*Hae*III fragment from KA-62 into the *Sma*I site of plasmid pGEM2. This subclone, referred to as pK5-3′, encompasses 190 bp of 3′ noncoding sequence, beginning at a position 44 nucleotides 3′ of the TAA stop codon. To make cRNA probes, pK5-3′ was linearized with *Bam*HI and transcribed by using SP6 RNA polymerase.

Genomic blots. Total human DNA (20 μg per sample) from blood leukocytes was digested with the indicated restriction endonucleases. Digested DNAs were separated by electrophoresis through 0.8% agarose and transferred to nitrocellulose paper by blotting (62). Radiolabeled cRNA probe to pK5-3′ (10^6 cpm/ml) was hybridized with the blot for 48 h at 42°C as described previously (46). After hybridization, blots were washed three times in 0.1% sodium dodecyl sulfate (SDS)–0.0015 M sodium citrate–0.015 M NaCl at 60°C, treated briefly with 0.1 μg of RNase per ml, and exposed to X-ray film for 7 days.

DNA sequencing. Plasmid pBluGK5-1 was prepared by subcloning a 7.9-kbp *Bam*HI fragment of GK5-1 into the Bluescript vector KS$^+$ (Stratagene, La Jolla, Calif.). This fragment contained 908 bp of flanking sequence 5′ of the ATG translation start codon and ~1,300 bp of flanking sequence 3′ of the TAA stop codon. DNA sequence analysis of pBluGK5-1 was conducted by the dideoxy method of Sanger et al. (59) and the double-stranded sequencing procedure described by Chen and Seeberg (4). A T7 promoter primer (20-mer; Promega Biotec) was used to determine the sequence of the 5′ end of pBluGK5-1. The remainder of the GK5-1 insert was sequenced by using 17-mer oligonucleotide primers synthesized with a model 380B DNA synthesizer (Applied Biosystems, Foster City, Calif.) and β-cyanoethyl chemistry.

Primer extension analysis. Total RNA from SCC13 cells was prepared by the method of Chomczynski and Sacchi (4a). The transcription initiation site of the human K5 gene was determined essentially as described by McKnight and Kingsbury (47a). An oligonucleotide primer 5′-CTTGAC TGGCGAGACAT-3′ was radiolabeled at the 5′ end by using [γ-^{32}P]ATP and T4 polynucleotide kinase. Labeled primer (5 × 10^4 cpm) was hybridized with 50 μg of total RNA from SCC13 cells. The DNA primer in the resulting DNA-RNA duplex was then extended by using reverse transcriptase. The RNA was degraded with pancreatic RNase A, and the extended DNA product was resolved on a 6% polyacrylamide sequencing gel. The primer extension products were compared with products of dideoxy sequencing reactions, using pBluGK5-1 as template and 5′-CTTGACTGGC GAGACAT-3′ as primer. The sequencing gel, containing both samples, was fixed in 10% acetic acid–5% ethanol, dried onto 3MM paper (Whatman, Inc., Clifton, N.J.), and exposed to X-ray film for 1 day.

Nuclear runoff assays. Relative transcriptional rates were measured essentially as described by Groudine et al. (24), with modifications suggested by Greenberg and Ziff (23). Briefly, nuclei from cultured human epidermal cells, WI38 human lung fibroblasts, cultured murine epidermal cells, and NIH 3T3 cells were isolated and stored at −70°C until assayed. Nuclei were thawed and incubated for 30 min at 30°C in the presence of transcription buffer (30 mM Tris hydrochloride [pH 8.0], 5 mM $MgCl_2$, 0.15 M KCl, 0.5 mM each ATP, CTP, and GTP, 2.5 mM dithiothreitol, 20% glycerol, 100 μCi of [^{32}P]UTP [800 Ci/mmol]), which allows elongation of RNA transcripts that were in the process of being formed at the time nuclei were isolated. Radiolabeled transcripts were then isolated and hybridized to denatured, nitrocellulose-immobilized DNAs containing keratin, actin, and tubulin cDNA sequences. Nitrocellulose filters were washed for 2 h at 65°C with two changes of 2× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]) and treated with 1 μg of RNase per ml of 2× SSC, followed by a final wash for 1 h in 2× SSC.

Construction of plasmids used for transfections. (i) K5 constructs. Plasmid pBluGK5-1 was prepared as outlined above. To prepare plasmid pJ2GK5-1, the 7.9-kbp *Bam*HI fragment of GK5-1 was cloned in the 5′-to-3′ direction into the *Bam*HI site of the polylinker region of plasmid pJay2 (19, 40), which contains a simian virus 40 (SV40) enhancer 5′ of the *Bam*HI site.

(ii) K6 and K7 constructs. Construction of plasmid pJK6b, containing the K6b gene driven by the SV40 major early promoter and enhancer, was described previously (19). Plasmid pJK7 was prepared by taking a 2,100-bp fragment containing the complete K7 cDNA sequence and subcloning it into pJay1, containing the SV40 major early promoter and enhancer (G. J. Giudice and E. Fuchs, unpublished results). The 2,100-bp fragment was obtained by ligating a 1,250-bp *Sac*I-*Eco*RI fragment from the cDNA KC-2 (21) to an 850-bp *Eco*RI-*Sac*I fragment (in the 5′-to-3′ orientation) from the genomic subclone pGK7.4 (20). This fragment contained 75 bp of sequence 5′ of the ATG start codon and ~700 bp 3′ of the TGA stop codon.

(iii) K14 constructs. Construction of plasmid pJK14, containing the human K14 gene driven by the SV40 major early promoter and enhancer, was described previously (19). A mouse K14 3′ noncoding plasmid was constructed by subcloning a 294-bp *Hin*dIII fragment of a mouse cDNA plasmid, pKSCC52 (34), into plasmid KS$^+$ (Stratagene). To make a 138-nucleotide probe complementary to the 3′ noncoding segment of mouse K14 mRNA, the plasmid was lineared with *Fsp*I and transcribed with T7 RNA polymerase.

Preparation of cell cultures. Mouse NIH 3T3 fibroblasts

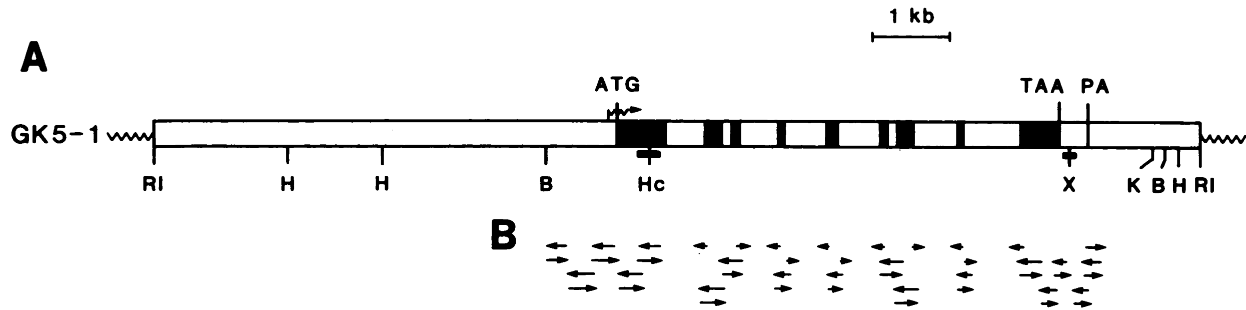

FIG. 1. (A) Map of the GK5-1 genomic clone. Clones were isolated from a human genomic library as indicated in Materials and Methods. Clone GK5-1 hybridized strongly with K5-5′, a subcloned segment of the 5′ end of K5 cDNA, represented by the bar encompassing the *Hin*cII (Hc) site. Clone GK5-1 also hybridized with K5-3′, a subcloned portion of the 3′ end of the K5 cDNA, represented by the bar encompassing the *Xba*I (X) site. Clone GK5-1 was partially mapped by restriction enzyme digestion and Southern blot hybridization (62). The restriction endonuclease sites used for subcloning fragments for chromosomal DNA analysis and sequencing are indicated. Abbreviations for other restriction endonucleases: RI, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I. Symbols: ~, phage arms; ■, the nine exons of the human K5 gene. The transcription initiation site was determined by using primer extension analysis as described in Materials and Methods. The major site is marked (⇝). Additional abbreviations: ATG, translation initiation codon of the K5 mRNA; TAA, translation termination codon of the K5 mRNA; PA, polyadenylation signal. (B) Sequencing strategy. Arrows indicate the direction and extent of the DNA sequence determined for each primer. All sequencing was conducted as described in the Materials and Methods, and the sequences for all exons, exon-intron borders, and 5′ and 3′ flanking regions were determined for both strands. Introns were not sequenced in full; except for introns II and VI, sizes are only approximate.

and WI38 human diploid fibroblasts (American Type Culture Collection, Rockville, Md.) were maintained in a 3:1 mixture of Dulbecco modified Eagle medium and Ham F12 medium supplemented with 10% newborn calf serum. Human and murine epidermal cells were cultured in the same medium supplemented with 10% fetal calf serum, 10^{-10} M cholera toxin, 5 ng of epidermal growth factor per ml, 10^{-11} M insulin, 2×10^{-10} M triiodothyronine, and 5 μg of human transferrin per ml. Epidermal cells also required a fibroblast feeder layer for growth. For immunofluorescence, cells were grown on chamber slides (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.).

DNA transfections. Plasmid DNAs were purified by CsCl density gradient centrifugation. All DNAs for transfection were subsequently dialyzed, extracted with phenol, and precipitated once with 2.5 M ammonium acetate–2.5 volumes of ethanol and once with 0.2 M NaCl–2.5 volumes of ethanol. After precipitation and washing, DNAs were stored at 1 mg/ml in 10 mM Tris hydrochloride (pH 7.9)–1 mM EDTA. By using a modification of the calcium phosphate precipitation method of Graham and van der Eb (22) and a 15% glycerol shock step (53), cells were transfected with 15 μg of keratin gene DNA plus 30 μg of KS^+ carrier DNA (Stratagene) per 10^6 cells. Cells were assayed at 65 h posttransfection.

Antibodies and immunofluorescent microscopy. Cells were grown on glass cover slides as described above. After transfection, cells were washed with phosphate-buffered saline, fixed in methanol (−20°C) for 15 min, and then washed again in phosphate-buffered saline. To detect expression of K5 and K6 proteins, we used a human type II epidermal keratin antiserum (15). For detection of the mouse K14, we used a monospecific K14 antiserum raised against a synthetic 15-amino-acid peptide specific for the carboxy termini of both mouse and human K14 (65). For general type I keratin detection, we used a polyclonal antiserum raised against gel-purified K14 (15).

To visualize primary antibody binding, we used fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (Organon Teknika, Malvern, Pa., and Tago, Burlingame, Calif.) as a fluorescent-labeled secondary antibody.

RESULTS

Isolation of a genomic clone containing a human K5 gene. A human genomic library in lambda phage Charon 4A was screened with radiolabeled probes to the 5′ and 3′ sequences of a human K5 cDNA (see Materials and Methods). One of these clones, GK5-1, hybridized with both the 5′ and 3′ cDNA probes and was chosen for further study. Analyses using restriction endonuclease digestions and Southern blotting (62) indicated that this clone contained a complete K5 gene with ~5,000 bp of 5′ and ~1,900 bp of 3′ flanking sequence (Fig. 1).

To determine whether there is a single K5 gene with this 3′ noncoding segment within the human genome, leukocyte DNA was digested with restriction endonucleases *Bam*HI, *Eco*RI, *Hin*dIII, and *Kpn*I, and the fragments were subjected to Southern blot analysis (62). Hybridization with a ^{32}P-labeled 3′ noncoding probe (GK5-3′) revealed the presence of single hybridizing bands for each digest (not shown). The mobilities of these hybridizing fragments corresponded to that expected from the GK5-1 clone, suggesting that the K5 gene contained within the GK5-1 clone is present in the human genome as a single species.

The sequence of the gene contained in GK5-1 corresponds perfectly to the sequence of a human keratin K5 cDNA. To verify that the isolated K5 gene was indeed the gene corresponding to the K5 cDNA cloned from human epidermal mRNA, the entire coding portion, exon-intron junctions, and 5′ and 3′ flanking regions of the gene were sequenced by the strategy shown in Fig. 1. Figure 2 illustrates the structure of the K5 gene, aligned with that of the human K6b epidermal type II gene, whose sequence was determined previously (70).

The sequences of the coding and noncoding segments matched perfectly with corresponding sequences previously reported for partial human K5 cDNAs (10a, 17, 43) and for partial K5 genomic DNA (10a). The predicted size of the encoded K5 protein was 61,456 Da, which is approximately 3,500 Da larger than that estimated from SDS-polyacrylamide gel electrophoresis (67).

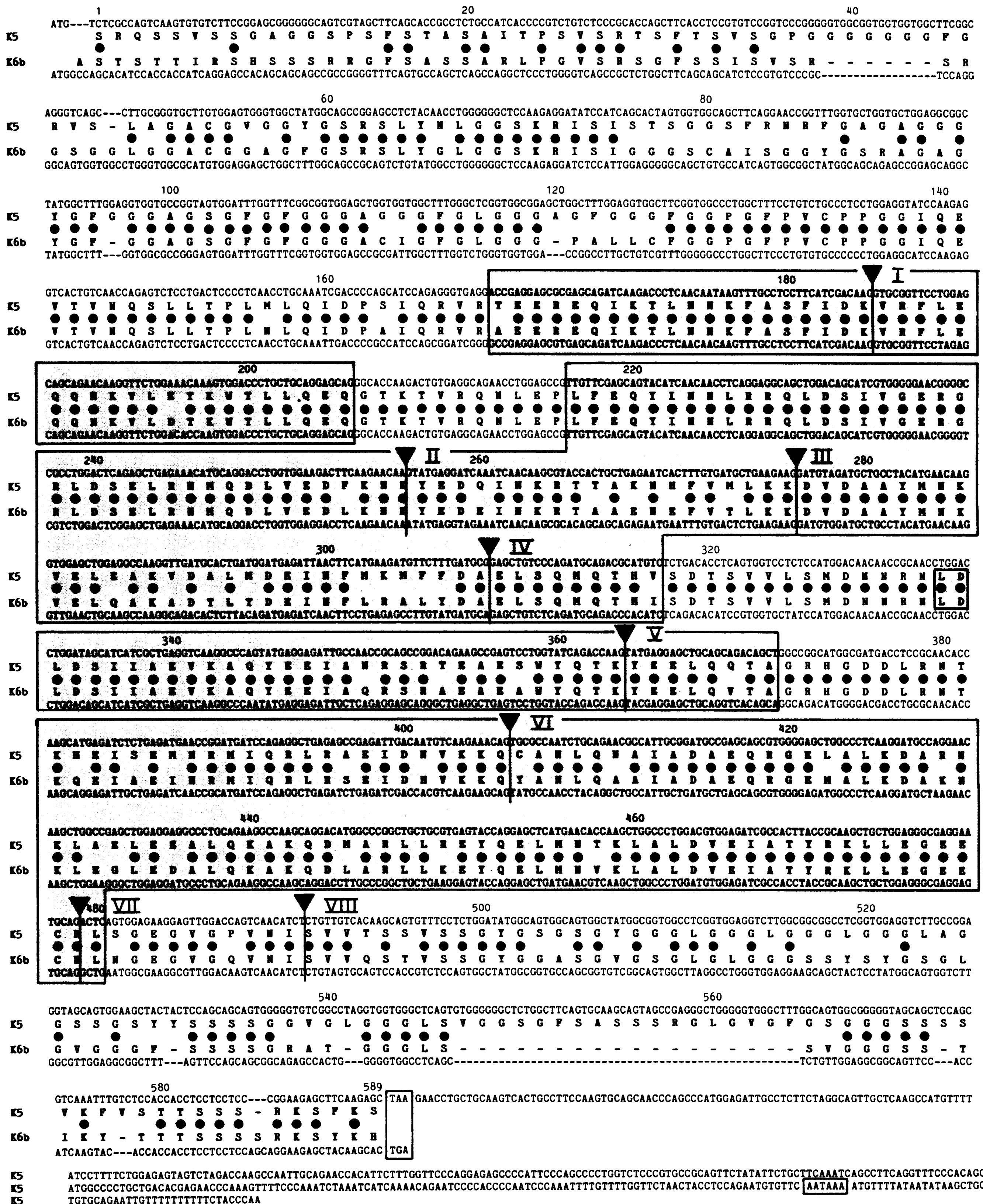

FIG. 2. Nucleotide and predicted amino acid sequences of the coding and 3′ noncoding regions of the type II human keratin gene contained in the genomic clone GK5-1. The coding and 3′ noncoding sequences of the human K5 gene are shown with 144 nucleotides per line. The sequence is shown aligned with the coding portion of a human epidermal type II keratin gene encoding K6b (70). Symbols: ●, amino acid residues identical for K5 and K6b (the two sequences show 78.1% identity at the amino acid level and 82.5% at the nucleic acid sequence level); ▼, intron positions. Exons were identified by comparing the sequence of the K5 gene with that of a K5 cDNA sequenced previously (43). The gray boxes mark the four alpha-helical domains in the K5 keratin. Throughout these domains are the heptad repeats of hydrophobic residues which identify the portions of the polypeptide that are involved in the coiled-coil interactions with a second keratin (16, 64).

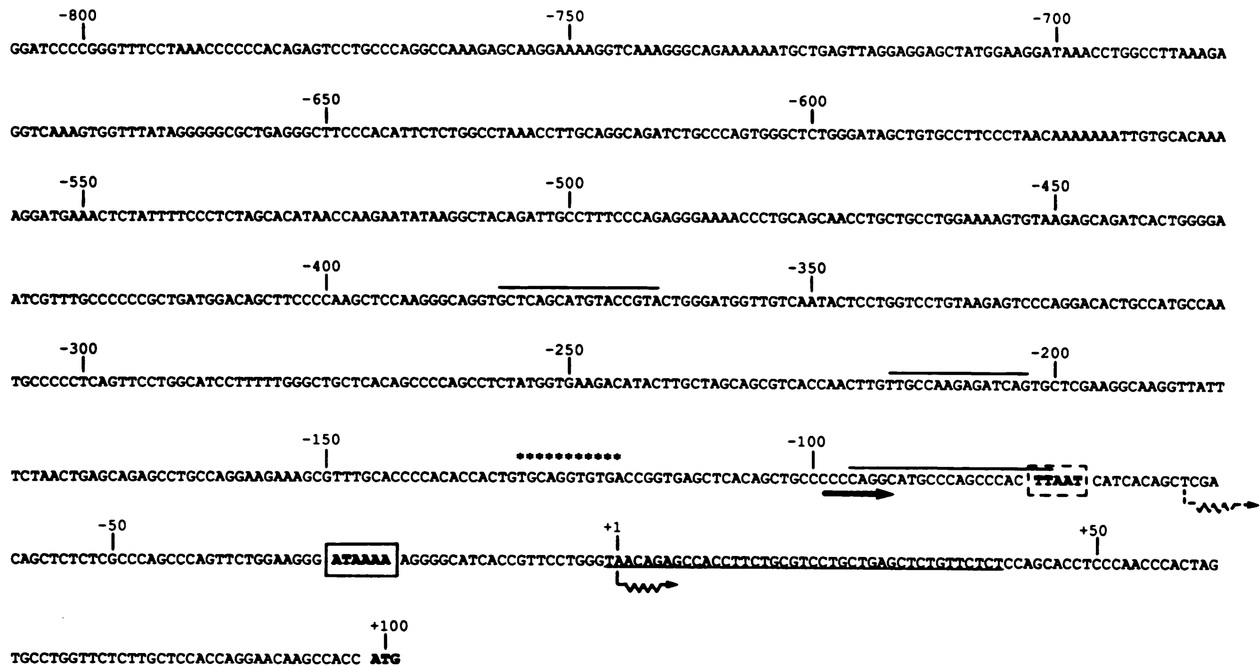

FIG. 3. 5′ Upstream region of the K5 gene. The 5′ upstream sequence of the K5 gene is shown beginning at 910 nucleotides 5′ upstream of the ATG translation initiation codon. The major TATA (ATAAAA) box is indicated by a solid box, and the major transcription initiation start site is marked by a solid wavy arrow. An infrequently utilized transcription initiation site suggested by the primer extension analysis in Fig. 4 is marked by the broken wavy arrow, and a possible corresponding TATA (TTAAT) box is indicated by the dashed box. A possible AP2-binding site (CCCCAGGC) is marked by the straight arrow. An 11-bp sequence sharing 100% identity with the type II K6b epidermal keratin gene is indicated (*), and three sequences sharing at least 75% homology with a corresponding segment of the type II K1 epidermal keratin gene are marked by overhead bars. Sequences in the 5′ noncoding portion of the K5 gene, which share 76% homology with the corresponding region of the partner type I K14 gene (47), are underlined.

In our earlier study, the structural similarities among members of the type II keratin protein family were demonstrated by aligning the sequence of the K5 cDNA with the coding sequences of the K6b gene (21). In this paper, the similarities in gene structure are shown by aligning the coding portions of the K5 gene with the corresponding regions of the K6b gene (Fig. 2). All eight intron-exon junctions of the K5 and K6b genes are identically positioned. This result was predicted by the fact that even the simple epithelial type II keratin gene K7 has intron-exon junctions that are nearly identical in position to those of the epidermal type II keratin genes (20). Furthermore, as noted previously for human epidermal type II keratin genes (29, 70), the intron-exon junctions did not seem to demarcate the structural boundaries of the alpha-helical domains of the keratin protein, predicted by computer methods (5, 6, 26, 27). The four alpha-helical segments of the K5 polypeptide are shown in Fig. 2. Throughout these domains are heptad repeats of hydrophobic residues, characteristic of all IF proteins and other alpha-helical polypeptides that form coiled-coil structures (18, 48, 49, 54).

Possible regulatory sequences for the human K5 gene. The 5′ upstream sequence of the K5 gene is shown in Fig. 3. At 124 nucleotides 5′ upstream of the putative translation initiation site, the sequence ATAAAA was found. Primer extension analysis revealed a transcription initiation site at 23 nucleotides 3′ of this sequence, suggesting that this sequence is utilized as a functional TATA box (Fig. 4). The overall size of the mRNA encoded by the GK5-1 gene is predicted to be 2,200 nucleotides, in good agreement with the previously determined size of the K5 mRNA (13, 43).

Of the core DNA sequences known to bind specific eucaryotic transcription factors, only one sequence was found to be of potential interest. This is the sequence CCCCAGGC (nucleotides −99 to −92), which shares 100% identity with other sequences known to bind the transcription factor AP2 (31, 42). Surprisingly, neither strand encompassing 807 nucleotides 5′ upstream of the transcription initiation site had any other sequences identical to known transcription factor-binding sites.

A comparison of 250 bp 5′ upstream of the K5 and K14 transcription initiation sites revealed no regions of substantial homology. However, the 5′ upstream sequences of other type II epidermal keratin genes did show some similarities. In particular, an 11-bp sequence (nucleotides −130 to −120) shared 100% identity with a 5′ upstream sequence in a K6b epidermal keratin gene, and three sequences shared at least 75% identity with a corresponding segment of the K1 epidermal keratin gene (Fig. 3). The significance of these homologies, if any, remains to be elucidated.

Interestingly, substantial sequence similarities were seen in the 5′ noncoding portions of the K5 and K14 mRNAs. In addition to the first 5 to 8 nucleotides at the 5′ and 3′ ends of the 5′ noncoding segments, there was a central stretch of 32 nucleotides with 76% identity (Fig. 3). This stretch also shared homology with the corresponding regions of other type II epidermal keratin genes (29, 70).

Regulation of expression of K5 and K14 at the transcriptional level. Although a comparison of several hundred nucleotides of 5′ upstream sequences of coordinately expressed sets of genes can sometimes provide useful insights into the molecular basis for gene regulation (31, 32), no obvious candidate sequences were obtained from our analyses of the human K5 and K14 genes. As a second step in

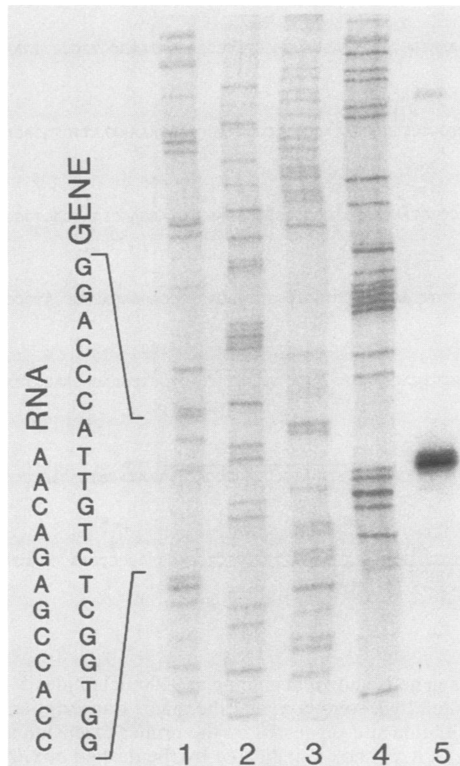

FIG. 4. Mapping of the transcription initiation site of the human K5 keratin gene. A synthetic 17-mer cDNA, 5'-CTTGACTGGC GAGACAT-3', complementary to the 5' end of the coding sequence of human K5 keratin mRNA, was 5' end radiolabeled with [^{32}P]ATP. This primer was hybridized with total RNA isolated from SCC13 cells and extended by using reverse transcriptase. After primer extension, the RNA was degraded with RNase A, and the extended DNA products were resolved on a DNA sequencing gel. Dideoxy sequencing reactions using pBluGK5-1 as template and the same primer as noted above were analyzed in parallel; therefore, a direct correlation can be made between the transcription initiation site and its position on the sequencing ladder. Lanes: 1 to 4, A, C, G, and T sequencing reactions, respectively; 5, primer extension products. K5 RNA sequence is in the 5'-to-3' direction and is shown at the left along with the corresponding K5 gene sequence in the 3'-to-5' direction. A small percentage (<5%) of the primer extension products suggested that an additional upstream initiation site may be utilized infrequently. This band is faintly visible at the top of lane 5, and its position is marked on the upstream sequencing diagram in Fig. 3.

exploring the mechanism of coordinate regulation of these keratins, we conducted nuclear runoff assays to determine whether the dramatic differences in the levels of K5 and K14 proteins in stratified squamous epithelial versus nonepithelial cells were reflected at the transcriptional level.

[^{32}P]UTP-labeled transcripts were prepared from cultured human epidermal cells, in which the levels of K5 and K14 are high, and from human diploid fibroblasts, in which K5 and K14 are not detectable. Conditions were chosen such that elongation, but not reinitiation, of transcripts was heavily favored (see Materials and Methods). When radiolabeled transcripts were hybridized to denatured DNAs immobilized to nitrocellulose, the levels of hybridization with the keratin cDNAs were several orders of magnitude greater for transcripts generated from epidermal nuclei than for those generated from fibroblast nuclei (Fig. 5). In fact, the levels of K5 and K14 transcripts in epidermal nuclei were significantly higher than for human α-tubulin and were comparable to levels for β-actin. In contrast, the relative levels of K5- and K14-hybridizing transcripts in fibroblast nuclei were extremely low, although upon longer exposure these signals seemed to be slightly higher than the background signals obtained with bacterial plasmid DNA (pGEM). Hence, these data indicate that at least a portion of the regulation of keratin expression in epidermal and nonepithelial cells is at the transcriptional level.

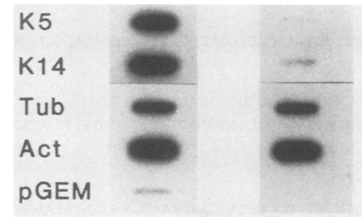

FIG. 5. Expression of K5 and K14 in human epidermal cells, but not fibroblasts, is reflected by cell-specific differences in transcriptional rates of the keratin genes. Nuclei were isolated from cultured human epidermal cells (A) and from human diploid fibroblasts (B) as described in Materials and Methods. Nuclei were incubated in the presence of [^{32}P]UTP under conditions that favored elongation (see Materials and Methods). Radiolabeled transcripts were then extracted and hybridized with denatured plasmid DNAs immobilized to nitrocellulose paper. After hybridization, the paper was washed and exposed to X-ray film for 3 (A) and 8 (B) days. Plasmid DNAs were as follows: K5, a 1.3-kbp human K5 cDNA in plasmid pGEM2 (43); K14, a complete human K14 cDNA (1.6 kbp) in plasmid pSP64 (the K14 sequence extends from the *Ava*I site of the K14 gene, GK-1, to the *Stu*I site of the K14 cDNA, KB-2 [13; Giudice and Fuchs, unpublished data]); Tub, a 1.4-kbp *Pst*I fragment of a human α-tubulin cDNA (7) in pGEM1; Act, an 819-bp *Pst*I fragment of a human β-actin cDNA (27) in pGEM1; and pGEM, control plasmid pGEM3-Z (Promega Biotec).

Appearance of endogenous K14 in NIH 3T3 fibroblasts transfected with the human K5 gene. Previous studies have demonstrated that transient transfection of a human K6b gene into mouse NIH 3T3 fibroblasts leads to the formation of filamentous structures, even though no single keratin by itself is sufficient for filament formation (19). Further analyses revealed that an endogenous type I epidermal keratin appeared in the cytoplasm of 3T3 cells as a consequence of K6b expression (19). At that time, however, sufficient tools were not available to (i) determine whether the type I keratin was K16, the corresponding partner to K6, or (ii) examine the mechanism of induction. Having the tools to explore this phenomenon in more detail, we first investigated whether the human K5 gene, in addition to the human K6 gene, had this inductive capacity.

The structure of the K5 expression vector, plasmid pJ2GK5-1, containing the GK5-1 gene and the SV40 enhancer sequences, is shown in Fig. 6A. When pJ2GK5-1 was transiently introduced into NIH 3T3 fibroblasts, transfected cells showed distinct filamentous structures which stained with an anti-type II epidermal antiserum (Fig. 7A). These networks were often perinuclear and wisplike and were indistinguishable from those obtained when 3T3 cells were transfected with pJK6b, containing the type II K6b keratin gene (Fig. 7 frame B; see Fig. 6C for details of the construct). The filamentous structures were distinctly different from the aggregates of keratin seen when NIH 3T3 cells were transfected with a type I K14 gene (Fig. 7C; see Fig. 6D for details of the construct and reference 19 for similar results). Collec-

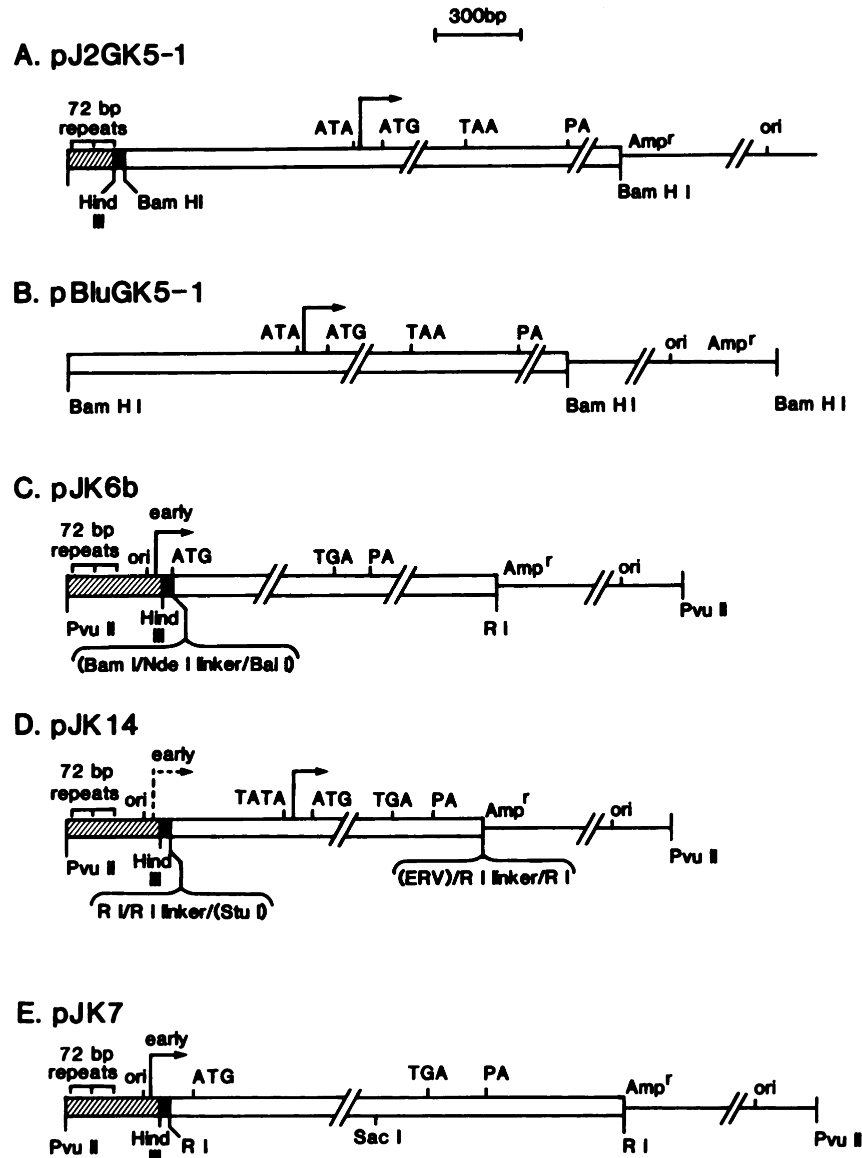

FIG. 6. Construction of K5, K6, K14, and K7 clones used for gene transfections. A detailed description of the preparation of these hybrid gene expression vectors is given in Materials and Methods. Symbols: ——, plasmid vector sequences (pJay2 [A; 40] pBluescript [B; Stratagene], or pJay 1 [C to E; 19]); ▭, human genomic sequences (A to D) or cDNA sequences (E); ■; polylinker sequences; ▨, SV40 sequences, including the 72-bp repeats (A) and the 72-bp repeats and major early promoter (C to E). The most frequently utilized transcription initiation sites (↱) and the less utilized sites (⇢) are indicated. The following keratin sequences were used in the plasmid constructions: the K5 gene from GK5-1 (A and B); the type II K6b gene (19) (C), the type I K14 gene (19) (D), and the type II K7 cDNA (20) (E). Abbreviations: ATA and TATA, recognition sequences for the binding of the transcription initiation factor; ATG, the translation initiation codon; TGA and TAA, translation termination codons; PA, polyadenylation signal. Abbreviations for restriction endonuclease sites: ERV, *Eco*RV; RI, *Eco*RI.

tively, these data indicated that K5 and K6b behaved in similar fashions and that both produced filamentous structures when expressed transiently in NIH 3T3 cells.

Since we had a monospecific antiserum raised in rabbits against a 15-amino-acid synthetic peptide analogous to the carboxy terminus of human K14, and since this sequence is shared by mouse K14, we were in a position to assess whether the filamentous structures detected in K5-expressing NIH 3T3 cells were due to the induced expression of the true partner for K5, namely, K14. Indeed, when pJ2GK5-1-transfected 3T3 cells were stained with the monospecific anti-K14 antiserum, the perinuclear filamentous networks were detected (Fig. 7D). Because we did not have a non-rabbit type II keratin antibody that cross-reacted with these filamentous structures, we were unable to conduct double-immunofluorescence studies. However, we did observe that these filamentous structures were indistinguishable from those detected with the rabbit anti-type II keratin antiserum (Fig. 7A). Mock-transfected control cultures showed no staining with either the type II or the monospecific anti-K14 antiserum. Collectively, these data suggested that expression of the type II keratin K5 resulted in the appearance of its true partner, K14, in NIH 3T3 cells.

To determine whether other type II keratins, such as K6b,

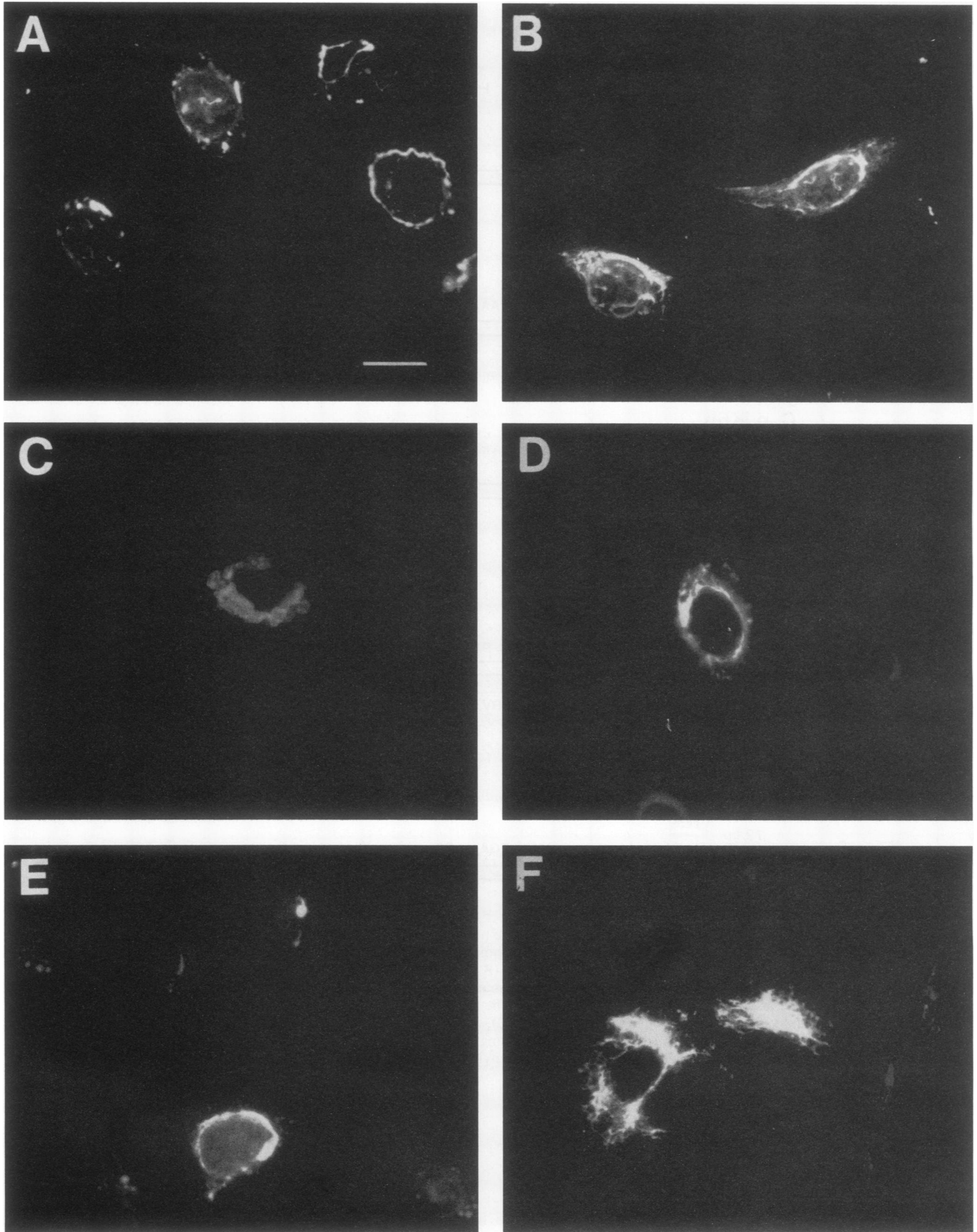

FIG. 7. Evidence that expression of K5, K6, and K7 in NIH 3T3 cells leads to the formation of perinuclear filamentous structures that costain with anti-K14 antiserum. NIH 3T3 cells were transfected with one of four plasmids: pJ2GK5-1 (A and D), pJK6b (B and E), pJK14 (C), and pJK7 (F). Details of the constructs are given in the legend to Fig. 6. At 65 h posttransfection, cells were fixed and subjected to indirect immunofluorescence. A general rabbit anti-type II keratin polyclonal antiserum (15) was used to detect the type II keratin gene products (A and B). To detect K14 induction (D to F) or K14 foreign gene expression (C), we used a rabbit polyclonal monospecific anti-K14 antiserum (65). Fluorescein-conjugated anti-rabbit secondary antibodies were used to visualize the rabbit antisera. Bar, 30 μm.

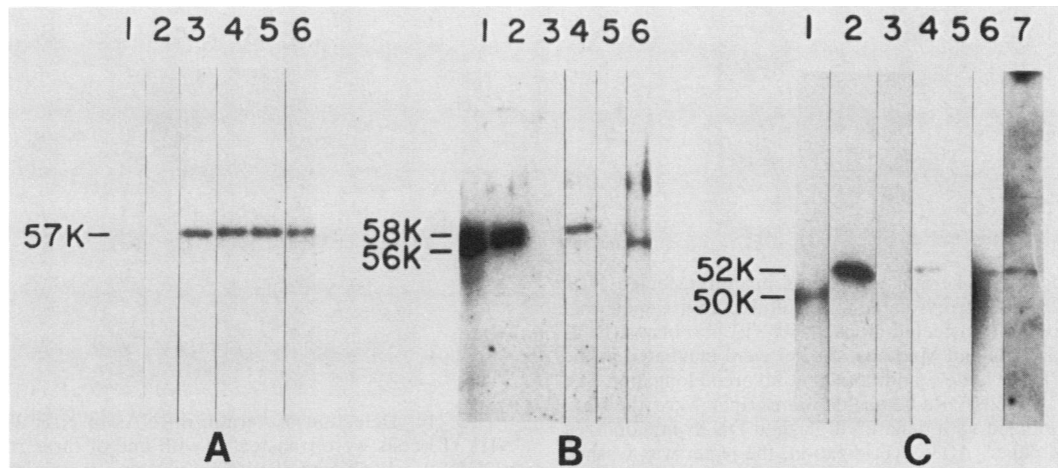

FIG. 8. Identification of mouse K14 induced in human type II keratin gene-transfected NIH 3T3 fibroblasts. NIH 3T3 cells were transfected with one of four plasmid DNAs: pJ2GK5-1, pBluGK5-1, pJK6b, and the Bluescript control vector KS$^+$ (Stratagene). The three constructs containing keratin genes are diagramed in Fig. 6. At 65 h posttransfection, cells were harvested and IFs were extracted (74). IF extracts (~2 μg for lanes 1 to 6; ~20 μg for lane 7) were resolved by SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose paper by blotting (69). Blots were incubated with a rabbit polyclonal antiserum against human vimentin (Stellmach and Fuchs, unpublished data) (A), a rabbit general anti-type II keratin antiserum (15) (B), a monospecific rabbit anti-K14 antiserum against the carboxy-terminal 15 amino acid residues of human K14 (65) (C), and a rabbit general anti-type I keratin antiserum (15) (not shown). Bound antibody was visualized by using ^{125}I-labeled *S. aureus* protein A. Panels A and B were exposed to X-ray film for 24 h before development. The lanes in panel C were exposed to X-ray film for 2 days (lanes 1 to 3), 24 h (lanes 4 to 6), and 13 days (lane 7). Extracts were from cells transfected with KS$^+$ (mock transfected) (lanes 3 and 7) pJ2GK5-1 (lane 4), pJBluGK5-1 (lane 5), and pJ2K6b (lane 6). IF extracts from untransfected cells were cultured human epidermal cells (lanes 1) and cultured mouse epidermal cells (lanes 2). Molecular sizes (in kilodaltons [K]) are indicated at the left.

also cause the induction of their true partner keratins in NIH 3T3 cells, we stained pJK6b-transfected cells with our anti-K14 antiserum. Since the true partner of K6 is K16 (11, 66), we were surprised to discover that the anti-K14 antiserum stained filamentous structures in pJK6b-transfected NIH 3T3 cells (Fig. 7E). Similar results were obtained with a construct (Fig. 6E) containing a cDNA encoding the human simple epithelial keratin K7, whose true partner seems to be the simple epithelial type I keratin K19 (66). pJK7-transfected NIH 3T3 cells showed marked staining with the anti-K14 antiserum (Fig. 7F). The finding that K7 expression still led to the appearance of K14 was even more surprising than the K6 induction: K6b shares 78.1% amino acid identity with K5, whereas K7 shares only 57.5% sequence identity.

Previous immunoblot analyses had shown that the anti-K14 antiserum was monospecific and did not cross-react with either human K16 or human K19. However, since the induced type I keratin was of mouse origin, we needed to verify that the immunofluorescence seen with the anti-K14 antiserum was indeed due to the presence of mouse K14 in the transfected cells. IF preparations were made from protein extracts of transfected and mock-transfected NIH 3T3 cells (Fig. 8). After electrophoresis through SDS-polyacrylamide gels and transfer to nitrocellulose by blotting (69), IF proteins were reacted with one of four antisera: antivimentin (V. Stellmach and E. Fuchs, unpublished results), anti-type II keratin antiserum (15), anti-K14 antiserum (65), and anti-type I keratin antiserum (15). As visualized by ^{125}I-labeled *Staphylococcus aureus* protein A, antibody binding to mouse vimentin was observed in all transfected and mock-transfected 3T3 cell extracts (Fig. 8A, lanes 3 to 6) but not in extracts from cultured human or mouse epidermal cells (lanes 1 and 2). In contrast, the anti-type II keratin antiserum detected K5 and K6 in both human and mouse epidermal cells (Fig. 8B, lanes 1 and 2, respectively). This antiserum also detected type II keratins in 3T3 cells transfected with type II keratin constructs (Fig. 8B, lanes 4 and 6) but not in mock-transfected cells (lane 3). In contrast to constructs containing the SV40 enhancer sequence, constructs without the enhancer did not yield detectable levels of keratin (lane 5). Hence, the SV40 enhancer sequences greatly elevated the level of transfected keratin gene expression.

The monospecific anti-K14 antiserum readily detected both human epidermal K14, which migrates at 50 kDa (67), and mouse epidermal K14, which migrates at 52 kDa (34) (Fig. 8C, lanes 1 and 2, respectively). In IF extracts from both pJ2GK5-1-transfected cells (lane 4) and pJK6b-transfected cells (lane 6), a 52-kDa band was detected with the anti-K14 antiserum. This band was not detected in the same amount of extract from mock-transfected NIH 3T3 cells (lane 3). These data indicated that endogenous mouse K14 was produced as a consequence of either K5 or K6b expression.

To determine whether any additional type I keratins might be induced upon K5 or K6b expression, we probed a final blot with a general anti-type I keratin antiserum (15). Even with a general antiserum, the only keratin detected in transfected 3T3 cells was the mouse K14 (data not shown). Hence, at least in NIH 3T3 fibroblasts, K14 seems to be promiscuous in its apparent inducibility by a mechanism that involves general type II keratin expression rather than specific expression of its bona fide partner.

The induction of K14 as a consequence of K5 expression in 3T3 cells does not appear to be regulated at the transcriptional level. To explore the molecular mechanisms underlying the appearance of mouse K14 in pJ2GK5-1-transfected 3T3 fibroblasts, we first determined whether untransfected 3T3 cells might transcribe the K14 gene even though they do not express stable K14 protein. Nuclear runoff assays revealed hybridization of radiolabeled 3T3 nuclear transcripts to nitrocellulose-immobilized K14 cDNA (Fig. 9B). The num-

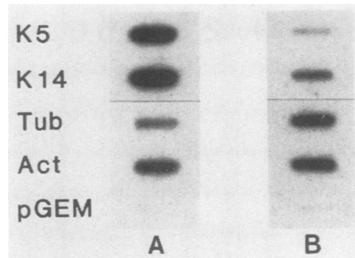

FIG. 9. Detection of keratin transcripts in untransfected NIH 3T3 fibroblasts. Nuclei were isolated from cultured mouse epidermal cells (A) and from untransfected mouse NIH 3T3 fibroblasts (B) as described in Materials and Methods. Nuclei were incubated in the presence of [^{32}P]UTP under conditions that favored elongation (see Materials and Methods). Radiolabeled transcripts were then extracted and hybridized with denatured plasmid DNAs immobilized to nitrocellulose paper. After hybridization, the paper was washed and exposed to X-ray film for 2 (A) or 3 (B) days. Plasmid DNAs were those indicated in the legend to Fig. 5.

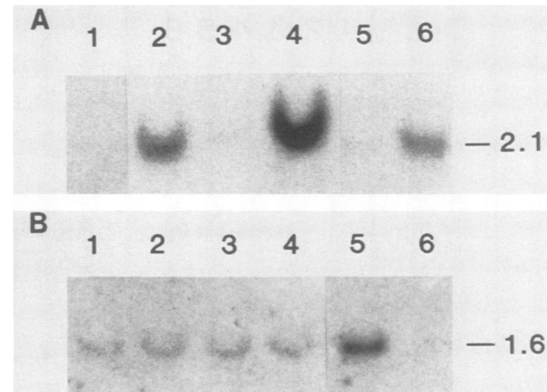

FIG. 10. Detection of keratin mRNAs in NIH 3T3 fibroblasts. NIH 3T3 cells were transfected with one of three plasmid DNAs: pJ2GK5-1, pJK6b, and pRSVK6b (a vector containing the K6b gene driven by the long-terminal-repeat enhancer and promoter of Rous sarcoma virus). At 65 h posttransfection, cells were harvested and RNAs were isolated as described previously (19). Samples of total RNAs (8 μg each) were resolved by formaldehyde-agarose gel electrophoresis and transferred to nitrocellulose paper by blotting (68). Two identical blots were then probed with a mixture of ^{32}P-labeled probes corresponding to the following sequences: pGEMK5, a 1,354-bp sequence complementary to a large portion of the coding region of K5 mRNA (43), and pSPK6, a 1,415-bp sequence complementary to the coding region of K6a mRNA (71) (A), and a 138-bp sequence corresponding to the 3′ noncoding portion of mouse K14 mRNA (B). This sequence was obtained by subcloning a *Hin*dIII-*Fsp*I fragment of kSCC 52, a 1,224-bp sequence complementary to a large portion of the coding and 3′ noncoding regions of mouse K14 keratin mRNA (34). After hybridization, each blot was subjected to RNase A (1 μg/ml) and extensive washing. Panels A and B were exposed to X-ray film for 5 and 14 days, respectively. Under these conditions, no background signals (e.g., from rRNAs) were detectable. RNA samples were from untransfected NIH 3T3 cells (lanes 1), NIH 3T3 cells transfected with pJ2GK5-1 (lanes 2), NIH 3T3 cells transfected (weakly) with pJK6b (lanes 3), NIH 3T3 cells transfected with pRSVK6b (lanes 4), cultured mouse epidermal mRNA (lanes 5), and cultured human epidermal mRNA (lanes 6). Sizes (in kilobase pairs) are indicated at the right. Under the conditions used, all type II keratin mRNAs from cultured human epidermal cells comigrated at 2.1 kbp (A), and all type I keratin mRNAs from cultured mouse and human epidermal cells comigrated at 1.6 kbp (B).

bers of 3T3 transcripts hybridizing to K14 cDNA were substantially greater than the number hybridizing either to K5 cDNA or to a bacterial plasmid control. Although hybridization of 3T3 transcripts to K14 cDNA was significantly less than that observed with transcripts from cultured mouse epidermal cells (Fig. 9A), the levels nonetheless suggested that the K14 gene was transcribed aberrantly in NIH 3T3 cells.

In a previous study, we used reduced-stringency conditions to detect hybridization between a radiolabeled human K14 cRNA probe and an NIH 3T3 mRNA that existed in both untransfected and K6b-transfected cells (19). Because of species differences, however, we were unable to confirm that a mouse K14 mRNA existed in untransfected NIH 3T3 fibroblasts. The availability of a mouse K14 cDNA (34) made it possible to determine unequivocally whether a stable mouse K14 mRNA existed in untransfected NIH 3T3 cells. The results of Northern blot analyses showed that an endogenous 3T3 mRNA of 1.6 kbp was detected with a radiolabeled cDNA probe complementary to a 3′ noncoding segment of mouse K14 mRNA (Fig. 10B, lane 1). The level of hybridization was comparable to that observed with mRNA isolated from pJGK5-1-transfected NIH 3T3 cells (Fig. 10A and B, lanes 2) and from pJK6b-transfected and pRSVK6b-transfected NIH 3T3 cells (lanes 3 and 4, respectively). Collectively, these data suggested that K14 mRNA existed in untransfected NIH 3T3 cells and that transfection of type II keratin genes into these cells did not greatly alter the level of this mRNA.

A priori, the dramatic difference in levels of K14 protein in untransfected versus K5-transfected 3T3 cells despite comparable levels in K14 mRNAs could reflect regulation at either the level of mRNA translatability or the level of K14 protein stability. Since type I and type II keratins are both involved in filament formation, it seemed most likely that K14 protein might be produced in 3T3 cells but unstable in the absence of a type II keratin partner. To determine whether K14 is expressed at low levels in untransfected 3T3 cells, we repeated our IF extractions and immunoblot analyses, this time using a 10-fold-greater amount of mock-transfected cell extract than was used previously. Anti-K14 detected a 52-kDa band in ~20 μg of IF extract from mock-transfected cells (Fig. 8, lane 7; 13-day exposure) but not in 2 μg of the extract (lane 3; 2-day exposure). The level of this protein was much less than that found in 2 μg of IF extract from pJ2GK5-1-transfected cells (lane 4; 24-h exposure). Given the differences in amount of extract loaded, exposure times, and the fact that only approximately 5% of the cells in pJ2GK5-1-treated cells were transfected, we estimate that the level of K14 protein in untransfected cells was ~100-fold less than in type II keratin-transfected 3T3 cells. Nevertheless, the fact that we could detect K14 in mock-transfected 3T3 cells indicates that at least a part of the endogenous K14 mRNA must be translatable. Thus, although we were unable to measure the turnover rate of the very low levels of K14 present in untransfected 3T3 cells, our data are best explained by the notion that type II epidermal keratin expression leads to a stabilization of endogenous type I K14 protein. This explanation for our epidermal keratin studies is consistent with recent findings by Kulesh et al. (38) for simple epithelial keratins.

DISCUSSION

Transcriptional regulation of keratins. A number of studies have demonstrated that the differential expression of kera-

tins is reflected in the relative levels of their mRNAs (33, 44, 57). However, there has been only one report demonstrating transcriptional control, and this was for F9 teratocarcinoma cells induced to differentiate in response to retinoic acid (52). Our nuclear runoff experiments provide the first experimental evidence of a case in which tissue-specific differences in keratin expression are attributed to changes in the transcriptional rates of the associated genes. Whether the coordinate expression of K5 and K14 involves a common transcriptional control mechanism remains to be elucidated. To this end, we were interested in examining the 5′ upstream sequences of the two genes.

A priori, since the sequences for a number of keratin genes are known (1, 3, 20, 29, 30, 37, 39, 46, 52, 55, 56, 58, 70), it would seem that sufficient data on comparisons of 5′ upstream sequences might already exist. Indeed, for a few cases, e.g., human K6 (70) and K16 (55, 58), the genes encoding the same species of true partner keratins have even been fully characterized. However, because of the large numbers of pseudogenes (39, 52, 58, 60) and multiple genes encoding keratins assigned a single number (71), it has been difficult to identify a single pair of keratin genes that are coexpressed in a certain epithelial cell and analyze whether there are sequence homologies which might contribute to this coordinate regulation. Our genomic analyses of the human K14 (46, 47) and human K5 (this paper) genes suggest that we have isolated and characterized the two genes which account for most, if not all, of the abundant keratin expression in the basal layer of stratified squamous epithelia in human cells. Hence, it provided us with a special opportunity to examine whether there might be similarities in the 5′ flanking sequences of these two genes.

Surprisingly, even though we analyzed 908 nucleotides of sequence 5′ of the ATG translation start site of the K5 gene and 318 nucleotides of 5′ sequence for the K14 gene, only a few similarities were uncovered, and these were predominantly in the 5′ untranslated sequences rather than the sequences residing 5′ of the transcription initiation site. Moreover, at least within the 908 nucleotides of 5′ upstream sequence that we provided here, there is no sequence identical to that proposed by Blessing et al. (3) as a tissue-specific regulatory element for epidermal cells, nor are there SV40 enhancer-like sequences similar to those present in the 5′ flanking sequence of the human K14 gene (47). In fact, our most striking finding is that the 5′ flanking regions of the human K5 and K14 genes seem to be highly dissimilar both to each other and to the counterpart regions of other previously sequenced human genes. As further investigations are conducted, however, we hope to be able to solve some of the mysteries still remaining concerning the sequences that play a role in the coordinate regulation of these two genes in stratified squamous epithelial tissues.

What is the mechanism of pairwise induction and is the in vitro induction of K14 physiologically relevant? While our nuclear runoff experiments indicated that transcriptional regulation is likely important in controlling the tissue-specific expression of the epidermal keratins, we were also interested in examining the molecular basis for the regulation of the balance of type II and type I keratins in epithelial tissues. Previously, we had demonstrated that the foreign expression of a type II keratin (K6b) in NIH 3T3 fibroblasts resulted in the apparent induction of an endogenous type I keratin and the subsequent formation of filamentlike structures (19). In the study described here, we extended these observations to include a second type II keratin, K5.

Our previous studies suggested that the accumulation of unpolymerized type II keratin is important in causing the appearance of type I keratin in fibroblasts, but we did not observe a reciprocal phenomenon with unpolymerized type I keratin, nor did we observed it in cells already expressing type II and type I keratins (19). Since keratins self-assemble in a 1:1 ratio of type I and type II proteins, a unidirectional mechanism of sensing an imbalance in this ratio could be an important step in correcting or readjusting the levels of type I and type II keratins in a cell. In the study reported here, the use of nuclear runoff and Northern blot assays using a specific probe for mouse K14 RNA enabled us to evaluate whether the sensory mechanism was at the transcriptional or posttranscriptional level. Our ability to detect K14-hybridizing transcripts and mRNAs in untransfected NIH 3T3 fibroblasts has now focused our attention on the possibility that posttranscriptional regulation is involved.

Our ability to detect K14 in untransfected NIH 3T3 cells at levels that are estimated to be 100-fold lower than in K5- or K6-expressing transfected cells suggests that K14 may be stabilized as a consequence of type II keratin expression. Because of the extremely low levels of K14 protein in untransfected 3T3 fibroblasts, we were unable to measure the K14 turnover rates in these cells and compare them with rates for either transfected 3T3 cells or epidermal cells. Hence, we cannot unequivocally rule out the possibility that some of the control is at the translational level. However, in this regard, recent studies on simple epithelial keratins have indicated that a type I keratin K18 is significantly less stable in cells lacking the complementary mouse type II keratin K8 than in cells that express both keratins (38, 39). Although simple epithelial keratins in general are significantly less stable than epidermal keratins, it seems likely that a common mechanism involving protein stabilization accounts for the balance of type II and type I keratins in a variety of different epithelial cell types.

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