Two Distinct Classes of Keratin Genes and Their Evolutionary Significance

Elaine V. Fuchs* and Susan M. Coppock
Department of Biochemistry
The University of Chicago
Chicago, Illinois 60637

Howard Green
Department of Physiology and Biophysics
Harvard Medical School
Boston, Massachusetts 02115

Don W. Cleveland
Department of Physiological Chemistry
Johns Hopkins University Medical School
Baltimore, Maryland 21205

Summary

Bacterial plasmids containing cDNA sequences specific for keratins were constructed from mRNA of cultured human epidermal cells. Two separate classes of cloned cDNAs were identified by positive hybrid selection: one class removed from total human epidermal mRNA a fraction that was translated into 50 and 55 kilodalton (kd) keratins, and the other class selected mRNAs that translated into a mixture of 50 kd and 46 kd keratins. When probes specific for these two keratin classes were hybridized with human DNA digested with a restriction endonuclease that does not cleave within the probe, two distinct patterns of about ten fragments each were observed. Most of the hybridizing genomic sequences, and it is estimated that each of the two classes is encoded by about 10 genes. When the probes were hybridized with DNA from different species, all vertebrates were found to contain discrete sequences homologous to both human keratin probes. Within each vertebrate species, the two probes always hybridized with approximately equal intensities to nonoverlapping sets of genomic sequences, suggesting a coordinate evolution between the two subfamilies of keratin genes. This finding has important functional implications for keratin filament assembly.

Introduction

The keratins are a family of at least 10 related proteins of 40 to 70 kilodaltons (kd) that form 80 Å cytoplasmic filaments in most vertebrate epithelial cells (Baden et al., 1973; Beckingham-Smith, 1973; Steinert and Idler, 1975; Huang et al., 1975; Reeves, 1975; Inoue et al., 1976; Dale et al., 1976; Culbertson and Freedberg, 1977; Brysk et al., 1977; Sun and Green, 1978a, 1978b; Franke et al., 1978a, 1978b; Fuchs and Green, 1978, 1980). Not all the keratins are synthesized simultaneously by any one cell; rather, different subsets of keratins are expressed during the course of terminal differentiation (Fuchs and Green, 1980) as well as in different epithelia (Doran et al., 1980; Fuchs and Green, 1980; Milstone and McGuire, 1981) and in different stages of development (Beckingham-Smith, 1973; Dale et al., 1976; S. Schlegel and H. Green, unpublished results).

To determine the extent to which individual keratins might be tailored to perform different structural or functional roles, various biochemical comparisons of the keratin polypeptides have been made. For the most part, these analyses have emphasized the similarities between the keratins. Proteolytic mapping has shown that the keratins differ to only a limited extent in primary sequence (Fuchs and Green, 1978). The keratins are also very similar in their amino acid compositions and immunological cross-reactivities (Baden et al., 1973; Steinert and Idler, 1976; Brysk et al., 1977; Sun and Green, 1978a; Fuchs and Green, 1978, Yuspa et al., 1980). A more sensitive and direct comparison of the keratins by amino acid sequence analysis has not been conducted.

We have begun to investigate the complexity of the keratin family through the use of bacterial plasmids containing DNA sequences complementary to keratin mRNAs of cultured human epidermal cells. Although we have previously demonstrated that each of the four major keratins produced by these cells is translated from its own mRNA (Fuchs and Green, 1979), we now show that these keratin mRNAs can be grouped into two distinct classes according to their nucleic acid sequences. Each class is encoded by about ten genes whose sequences do not cross-hybridize with members of the other class. Moreover, these two distinct gene classes appear to be maintained as separate, unlinked subfamilies throughout vertebrate evolution, suggesting that both classes are essential for the structure and function of the keratin filaments.

Results

Construction and Identification of Keratin cDNA Clones

Double-stranded cDNA was constructed from mRNA of cultured human epidermal cells and inserted into the Pst I site of E. coli plasmid pBR322 (as detailed in Experimental Procedures). The closed circular plasmids were subsequently used to transform E. coli X1776 according to the procedure of Kushner (1978). Of the selected tetracycline-resistant clones, 93% were ampicillin-sensitive, indicating that they contained a hybrid pBR322-cDNA plasmid. A total of 1000 tetracycline-resistant, ampicillin-sensitive clones were obtained from 200 ng of double-stranded cDNA.

The clones were screened (Grunstein and Hogness, 1975; Craig et al., 1979) for their ability to hybridize with 32P-labeled cDNA prepared from mRNA of cul-
tured human epidermal cells. Approximately 30% of this mRNA was comprised of sequences specific for the four major keratins of epidermal cells. Colonies that hybridized strongly to the $^{32}$P-labeled cDNA were likely to contain DNA sequences complementary to the abundant keratin mRNAs and were selected for further investigation.

Plasmid DNA was purified from several of these clones and used in a positive hybridization-translation assay (Ricciardi et al., 1979; Cleveland et al., 1980). Messenger RNA that specifically hybridized with the cloned cDNAs of individual plasmids was translated in vitro and analyzed by polyacrylamide gel electrophoresis and fluorography. The translation products of two representative clones identified as containing keratin sequences are shown in Figure 1, lanes 2 and 3. The products were identified as keratins by their molecular weights, their specific precipitation by antiserum against human s. corneum keratins and their one-dimensional polypeptide patterns generated by S. aureus V8 protease (data not shown). These methods of identifying the keratins have been described elsewhere (Fuchs and Green, 1978, 1980).

Of the initial clones chosen for assay, approximately 25% contained sequences complementary to the keratin mRNAs. Surprisingly, two distinct types of clones were identified. One class specifically selected mRNA coding for the 56-58 kd keratins (Figure 1, lane 2), whereas the other class specifically selected mRNA coding for the 46-50 kd keratins (Figure 1, lane 3). Of 20 clones tested, 3 belonged to the first class and 2 belonged to the second. Additional keratin clones identified later (see below) also selected either one or the other of the two classes of keratin mRNAs, but never both. Thus, though the four keratins of cultured human epidermal cells are similar (Fuchs and Green, 1978) they can be segregated into two groups by this assay.

Identification of Additional Keratin Clones by Back-Screening

The size of the keratin cDNA sequences contained in each of the five initially identified hybrid plasmids was determined by excising the inserted cDNA sequences with Pst I restriction endonuclease and separating the cDNA from the pBR322 DNA by electrophoresis on a 5% polyacrylamide gel. The largest of the three 56-58 kd keratin plasmids, pKA-1, contained a cDNA insert (KA-1) with four internal Pst I restriction sites, and hence upon Pst I digestion yielded five insert fragments of 560, 475, 430, 125 and 90 bases for a total length of 1680 bases. The largest of the two 46-50 kd keratin plasmids, pKB-1, contained an insert (KB-1) with a single internal Pst I site yielding two insert fragments of 555 and 510 bases for a total length of 1065 bases. These two plasmids were chosen for back-screening the library for additional clones bearing keratin sequences (Grunstein and Hogness, 1975).

A total of 11 clones containing sequences complementary to the KA-1 probe and 12 clones containing sequences complementary to the KB-1 probe were selected. Using positive hybridization-translation, we confirmed that all 23 of these newly identified clones could be segregated either into the class that selected mRNA translatable into the 56-58 kd pair of keratins.
or into the class that selected mRNA encoding the 46–50 kd pair. Even small clones of 600–750 bases selected the same classes of mRNAs as did the large clones.

None of the additional 56–58 kd keratin clones contained cDNA sequences longer than the original 1680 base insert of pKA-1; however, one plasmid, pKB-2, contained a 1450 base insert homologous to the original 46–50 kd keratin plasmid pKB-1 (1065 bases). KB-2 had two internal Pst I sites and gave three insert fragments of 590, 455 and 405 bases upon digestion with the enzyme. For the experiments described below, KA-1 and KB-2 were used as probes specific for the two classes of keratin cDNAs.

Size of mRNAs Hybridizing with the Cloned Keratin cDNAs
Poly(A)+ RNA from cultured human epidermal cells was fractionated by electrophoresis on an agarose gel containing 2.2 M formaldehyde (Boedtker, 1971). The RNA was transferred to nitrocellulose paper according to the procedure of Thomas (1980) and hybridized with either of the two keratin probes. RNA homologous to the probes was visualized by autoradiography (Figure 2). Lane 1 shows a single band of 2000–2200 bases that hybridized to the 56–58 kd keratin probe. Based on this size estimate, our largest cloned 56–58 kd keratin cDNA probe (KA-1) encompasses about 80% of the corresponding mRNA. Lane 2 of Figure 2 shows a single band of 1550–1650 nucleotides that hybridized to the probe for 46–50 kd keratin cDNA. Our largest 46–50 kd keratin cDNA insert (KB-2) therefore corresponds to about 90% of the total mRNA length. For both lanes, additional bands of hybridization were visible when the films were exposed for a longer period. The 56–58 kd keratin probe hybridized with bands of 4500 and 8000 bases, and the 46–50 kd keratin probe hybridized with bands of 3500 and 6500 bases. Although their functional significance is unknown, these large RNAs may represent unprocessed or partially processed nuclear transcripts.

Hybridization of Keratin cDNA Probes with Human DNA
Using the cloned cDNAs for the two keratin classes, we investigated their corresponding genetic complexities. High molecular weight human DNA was prepared from cultured epidermal cells and from placenta and digested with restriction endonucleases that did not cut within the sequences of either cloned keratin cDNA insert. We subjected 5 μg of each digest to gel electrophoresis. Adjacent lanes were loaded with an amount of unlabeled linearized keratin cDNA insert corresponding to that amount which would be present in 5 μg of human DNA if the sequence were present at 0.5, 1, 3, 5, 10, 25 or 50 copies per haploid human genome (3.3 x 10⁹ base pairs). The DNA was transferred to nitrocellulose paper (Southern, 1975) and hybridized with ³²P-labeled probe copied from the cDNA of each class of keratin insert.

Figure 2 shows the hybridization of the digested DNA with the labeled probes transcribed from the 56–58 kd keratin cDNA, KA-1, and the 46–50 kd keratin cDNA, KB-2. In both cases, a number of bands of
DNA (5 μg per lane) from cultured human epidermal cells was digested with a fivefold excess of each of three restriction endonucleases. Fragments were fractionated by electrophoresis through a 0.8% agarose gel and transferred to nitrocellulose paper (Southern, 1975). The blots were hybridized against 32P-labeled probes copied from KA-1, the 56-58 kd keratin cDNA (A), and KB-2, the 46-50 kd keratin cDNA (B). Lanes contain either human epidermal DNA (EPI) or human placental DNA (PLAC) digested with Hind III (H3), Eco RI (RI) or Barn HI (B). Additional lanes represent dilutions into 5 μg of sheared E. coli DNA of the appropriate linearized hybrid plasmid, either pKA-1 (A) or pKB-2 (B), in amount corresponding to 0.50 to 50 copies of keratin cDNA per haploid genome (see Experimental Procedures for details). Lane C shows a control in which the plasmid pBR322 was added in amount corresponding to 10 copies per haploid genome. Molecular weights in kilobases are shown at the left.

hybridization were observed. Depending on the endonuclease used, 8 to 12 fragments hybridized with KA-1. Most bands had an intensity corresponding to that expected for a gene present in one to three copies per haploid genome. Using a densitometer to scan the autoradiogram and sum the intensities of the bands in each track, we determined that sequences similar if not identical to those of KA-1 were repeated approximately ten times in the human genome.

KB-2 also hybridized with seven to ten restriction fragments (Figure 3B), but the pattern of hybridizing fragments selected by this probe was clearly different from that selected by KA-1. Scanning the intensity of these bands indicated that sequences hybridizing with KB-2 were also repeated about ten times within the human genome. Few if any of the DNA fragments hybridized to both KA-1 and KB-2, indicating that for the most part the two classes of keratin genes represent nonoverlapping sets of sequences which are not closely linked.

For both classes of keratins, the banding patterns were identical for placental and epidermal DNA. This demonstrates that no amplification or gross alteration of the keratin genes occurs during development. In addition, although samples of DNA from only two humans have been examined, the patterns of hybridization for these two samples were indistinguishable, suggesting that extensive heterogeneity due to allelic differences in the coding sequences (Hughes et al., 1979) is probably not present in the two keratin subfamilies.

When the autoradiogram shown in Figure 3 was exposed for a longer period, about 10 to 15 additional bands of 1–25 kb were seen in each lane of digested human DNA. The total intensity of these bands was probably less than that corresponding to a single copy of cDNA per haploid genome. These weak bands could represent either partial copies of the cDNA sequences or heterologous hybridization between similar, but not identical, sequences.

Hybridization of Genomic DNA with Probes Corresponding to the 5' and 3' Ends of the Keratin mRNAs

Figure 3 shows that a restriction endonuclease that does not cut within either keratin cDNA generates multiple fragments of human genomic DNA that hybridize with the keratin probes. To determine whether these fragments carry sequences homologous to both the 3' and 5' ends of either keratin probe and hence represent essentially complete genes, we repeated the hybridization experiments, using 32P-labeled probes copied from either the 3' end or the 5' end of the cloned insert.

To prepare the partial length probes from pKA-1, the plasmid was digested with a combination of Bam HI and Kpn I, generating two fragments containing cDNA: a large fragment containing roughly 1300 bases of the original insert and a small fragment containing approximately 380 bases of the insert. Treatment of pKB-2 with Bam HI and Sst I yielded two fragments containing approximately 550 and 900 base pairs of cDNA. Although we do not know which fragment of each pair corresponds to the 5' end of the mRNA and which the 3' end, this is not essential to the interpretation of the data presented here.

The restriction fragments of digested human DNA containing sequences complementary to each part of the 56–58 kd keratin cDNA are shown in Figure 4.
Epidermal Keratin Genes

Figure 4. Test for Complete 56–58 kd Keratin cDNA Sequence in DNA Restriction Fragments by Hybridization with Probes Complementary to the 3' and 5' Ends of the Cloned Insert

Aliquots (5 μg each) of human DNA were digested with a fivefold excess of each of three restriction endonucleases. Samples in triplicate were subjected to electrophoresis through a 0.6% agarose gel, then transferred to nitrocellulose paper. The paper was cut and hybridized against 32P-labeled probes copied from the segment of pKA-1 containing the 380 base end of the 56–58 kd keratin cDNA insert, generated by Kpn I·Bam HI digestion (A); the other segment of pKA-1, containing the 1300 base end of the insert (B); and linearized pKA-1 (C). Lanes marked H3 contain DNA digested with Hind III; lanes marked RI, with Eco RI; lanes marked B, with Bam HI. Molecular weights in kilobases are shown at the left.

The first lane of each set (Hind III digestion) shows that two major bands hybridized with both ends of the 32P-labeled probe, but a band of 7 kb hybridized only with the 380 base insert (Figure 4A), and a band of 6 kb hybridized only with the 1300 base sequence (Figure 4B). For the Eco RI and Bam HI digestions (lanes 2 and 3, respectively, of each set), most of the fragments hybridized with both ends of the probe. In a separate control experiment, no significant cross-hybridization occurred between either set of partial probes, eliminating internal homologous sequences as a possible cause of hybridization by both 5' and 3' ends of the fragment and consistent with the presence of the entire cDNA sequence in most of the DNA restriction fragments.

Similar results were found for the 46–50 kd keratin probe (Figure 5). As an example, the Hind III digestion (center lane of each set) showed that fragments of 12.9, 10.6, 9.5 and 5.7 kb hybridized with both ends of the probe, but fragments of 14.1 kb and 2.75 kb hybridized only with the 550 base insert (Figure 5A), and fragments of 4.7 kb and 3.25 kb hybridized only with the 900 base insert (Figure 5B).

The preponderance of fragments hybridizing with both ends of the keratin probes supports the suggestion that there are multiple genes for each of the two classes of keratins. The existence of fragments that hybridize only with part of the total keratin probe is difficult to interpret, but the following explanations are consistent with the existing data: the keratin genes may contain introns, some of which have Bam HI, Eco RI or Hind III restriction endonuclease sites; there may exist a population of heterologous genes that cross-hybridize to a single keratin cDNA clone, and some of these could possess restriction endonuclease sites not found in the cDNA of the probe; or some of the keratin genes may be incomplete pseudogenes (Miller et al., 1978; Firtel et al., 1979; Hardison et al., 1979; Vanin et al., 1980; Nishivka et al., 1980; Lauer et al., 1980; Denison et al., 1981).

Hybridization of Human Keratin Probes to DNAs of Other Species

The evolutionary relatedness of the keratins was examined by hybridizing the human keratin cDNAs with DNA from other species. DNA from yeast, drosophila, sea urchin, hagfish, angler fish, chicken, mouse and human was digested with Eco RI, subjected to electrophoresis through a 0.8% agarose gel, transferred to nitrocellulose paper and hybridized with 32P-labeled copies of the purified inserts, KA-1 and KB-2. Autoradiograms of the resultant blots are shown in Figure 6. For a given species, the DNA fragments recognized by the two keratin probes segregated into distinct, apparently nonoverlapping sets of sequences: one set that hybridized to KA-1 and one set that hybridized to KB-2. The number of hybridizing bands and their relative intensities were approximately the same for each of these two sets. The strongest cross-hybridization was observed with mouse DNA (Figure 6, lane 2), which showed seven to nine bands with either probe. Per microgram of DNA, the total intensity of hybridization with either probe was approximately 50% that of human DNA. The total intensity of hybridization for other vertebrate DNAs was less, with chicken DNA giving 20% (lane 3), angler fish DNA 15% (not shown), and hagfish DNA 10% (lane 4). Interestingly, hagfish DNA showed only one major band that hybridized with the 56–58 kd keratin cDNA probe (Figure 6A, lane 4) and only two bands that hybridized to the 46–50 kd probe (Figure 6B, lane 4). It is possible that these few keratin sequences in hagfish represent the primordial epidermal keratin genes prior to duplication and divergence in the higher
Vertebrates. In any case, these data show that the two classes of keratin sequences have been maintained in each of the vertebrate genomes.

Hybridization of either cDNA probe to DNA digestion products from lower eucaryotes showed no distinct banding patterns. Under stringent conditions, neither probe hybridized with DNA of drosophila (lane 5) or yeast (lane 6). Sea urchin DNA gave somewhat ambiguous results (not shown). After digestion with Eco RI, Hind III or Bam HI, two separate preparations failed to show discrete bands when hybridized with either keratin probe. Instead, diffuse hybridization was observed. This cannot be attributed to degradation of the DNA, since hybridization of the same filters with chicken \(\beta\)-tubulin cDNA probe showed discrete bands as expected (Cleveland et al., 1980). Whether the sea urchin genome contains a repetitive sequence that shares some homology with the keratin cDNAs remains to be explored.

**Discussion**

Of the three cytoskeletal components of epithelial cells, the keratins seem to have evolved more recently.
than the actins and tubulins. Amino acid sequence data have shown that the actins are very highly conserved from humans down to even the unicellular eucaryotes (Vandekerckhove and Weber, 1978). This conservation extends to the nucleic acid sequence as well (Kindle and Firtel, 1978; Gallwitz and Sures, 1980; Ng and Abelson, 1980). Some features of the tubulins also seem to have been preserved from the simpler eucaryotes to the higher organisms (Kilmartin, 1981), and strong complementarity at the level of the nucleic acid sequence extends from human to drosophila and sea urchin (Cleveland et al., 1980). Although the possibility that keratins might be present in lower eucaryotes has not been investigated, the complementarity of the nucleic acid sequence of the keratins extends only throughout the vertebrates, suggesting that the keratin filaments have evolved to meet the protective needs of the epidermis of soft vertebrate skin. Whether other intermediate filament proteins are also of relatively recent origin remains to be determined.

The conservation of the two groups of keratin sequences in vertebrates indicates that the evolutionary pressure exerted on each class of keratins has maintained at least some common regions of nucleic acid sequence relatively unaltered. This degree of conservation is initially surprising, since considerable divergence in the sizes of the keratin polypeptides within each group appears to have been tolerated. However, it must indicate restrictions on amino acid sequence for each keratin polypeptide that are essential for filament function.

Although the existence of two classes of keratins has not hitherto been fully recognized, it is consistent with earlier analyses of the keratin polypeptides. One-dimensional peptide mapping with S. aureus V8 protease and chymotrypsin showed greater differences between human epidermal keratins of different sizes than between those of similar sizes (Fuchs and Green, 1978). Distinctions between keratins of different sizes have also been made for other mammalian keratins (Huang et al., 1975, Steinert et al., 1976, 1978). Our findings make these differences obvious. The cloned cDNAs of these two classes clearly hybridize to different sets of keratin mRNAs and to different sets of human DNA fragments, confirming the existence of two different subfamilies of keratin genes.

There are about ten genes per keratin subfamily. We do not know whether the genes for other keratins not abundant in cultured epidermal cells are members of either class of keratin genes identified here, or whether they represent members of additional keratin subfamilies. However, it is interesting to note that the number of genes so far discovered for the keratin family is much smaller than the large number predicted for avian feather keratins in uncloned keratin cDNA hybridization studies (Kemp, 1975). A large number of feather keratin genes could be necessary for the rapid replacement of embryonic feathers with adult feathers, a developmental feature not paralleled for the epidermis. The feather keratins are very different from epidermal keratins in both size and amino acid composition (Kemp and Rogers, 1972), and their genes would not be expected to contribute to the cross-hybridization seen between the human keratin probes and chicken DNA in Figure 6.

Perhaps the most intriguing possibility raised by the documentation of two classes of keratins in vertebrates is that the two kinds of keratins may both be required for filament assembly. This suggestion is consistent with earlier studies by Steinert et al. (1976) and Lee and Baden (1976), who observed that formation of keratin filaments in vitro could not take place with only one keratin polypeptide, but that certain combinations of two purified keratins were sufficient. A requirement for two classes of keratins in filament assembly would explain the remarkable conservation of these two unlinked subfamilies of keratin genes throughout vertebrate evolution.

### Experimental Procedures

#### Preparation of Cell Cultures

Human epidermal cell strains were derived from the foreskin of newborns and used in their second to fourth subculture. They were grown in the presence of lethally irradiated 3T3 cells in fortified Eagle's medium supplemented with 10% fetal calf serum, 0.4 mg/ml hydrocortisone (Rheinwald and Green, 1976, 1977), 4 ng/ml epidermal growth factor prepared by the method of Savage and Cohen (1972), 10^{-10} M cholera toxin (Green, 1978), 2 x 10^{-10} M T3, 5 μg/ml human transferrin, and 6 μg/ml insulin (Barnoc and Eato, 1980; B. Schlegel and H. Green, unpublished results; Maciag et al., 1981). The medium was changed every 3 to 4 days, and also 12 hr before the cells were harvested.

#### Isolation of Poly(A)^+ RNA

Poly(A)^+ RNA was isolated from human epidermal cells three-quarters confluent in fifty 100 mm culture dishes according to the guanidine procedure of Strohman et al. (1977), modified as described by Fuchs and Green (1979).

#### Preparation of Double-Stranded cDNA

Double-stranded cDNA was prepared from 25 μg of human epidermal mRNA essentially according to the procedure of Wickens et al. (1978). We synthesized the first strand at 42°C for 60 min using reverse transcriptase (Beard, Life Sciences) in the presence of 170 mM KCl. We synthesized the second strand using DNA polymerase I (New England Dicloabe) at a final concentration of 50 units/μg of initial mRNA. Double-stranded cDNA was phenol-extracted and chromatographed on Sephadex G-75, and excluded cDNA was precipitated with ethanol as described by Ullrich et al. (1977). After 31 nuclelease treatment to break the hairpin loop (Ullrich et al., 1977), the double-stranded cDNA was tailed at the 3' termini with approximately 20 cytosine residues by use of terminal deoxynucleotidyl transferase (P-L Biochemicals) according to the procedure of Chang et al. (1978). The double-stranded cDNA of size 1000-3000 bp (about 3 μg total) was hybridized by using DNA in a Bio-Gel A-5m column. The appropriate region was pooled and ethanol-precipitated with 50 μg of yeast tRNA (Miles) added as carrier.

The size and tailed double-stranded cDNA was mixed at a weight ratio of 1:3 with plasmid pBR322 that had been linearized at the Pst I restriction site and tailed with approximately 15 to 20 guanosine residues. To anneal the guanosine-tailed plasmids to the cytosine-tailed double-stranded cDNA, the mixture at a concentration of 1 μg/ml total DNA was incubated at 65°C for 2 min and then incubated for 2 hr at 42°C, followed by 2 hr at 30°C and finally 1 hr at 14°C.
Transfer RNA was added to 20 μg/ml, and the sample was ethanol-precipitated.

Transformation of X1776

Hybrid DNA, annealed by the Ga-tailing method described above, was used to transform X1776 according to procedure of Kushner (1975).

Screening of Recombinant Clones

Recombinant clones were selected for tetracycline resistance (20 μg/ml in agar plates) and screened for ampicillin sensitivity (50 μg/ml in agar plates). Colonies responding appropriately to the drugs were screened according to the modification by Croce et al. (1979) of the colony hybridization protocol of Kafatos et al. (1978). Clones were spotted in an orderly fashion onto seven circular Whatman 1 filters placed onto 1 cm thick beds of nutrient agar. After the colonies were allowed to grow on the filters for 48 hr at 37°C, the DNA from each clone was denatured and fixed onto the filter. 32P-labeled cDNA was transcribed from total human epidermal mRNA (about 30% keratin mRNA) and hybridized (see below). Strongly hybridizing clones were identified by autoradiography on Kodak X-Omat AR-6 film with Du Pont CronexTM Lightning Plus intensifying screens.

Isolation of Plasmid DNA

Supercropped plasmids from strongly hybridizing clones were isolated and purified as follows. A 4 ml culture grown overnight in M9 minimal medium (Adams, 1959) at 37°C was transferred to 400 ml of the same medium supplemented with 10% L broth. Cells were grown to an absorbance of 0.850 at 650 nm. At this time, 10% L broth, 0.2% casamino acids and 0.2% glucose were added, and the cells were grown an additional 30 min at 37°C. Chloramphenicol was added to 170 mg/l, and the cells were left with constant shaking for 12 hr at 37°C. Cells were harvested by centrifugation (5500 rpm in a GS-3 rotor for 15 min), washed twice in 10 mM NaCl, 10 mM Tris (pH 7.0), then frozen at −70°C for 30 min. Cells were resuspended in a solution of 25 mM Tris (pH 8.0), 10 mM NaEDTA and 50 mM glucose containing 2 mg/ml lysozyme (Sigma) and allowed to incubate for 15 min at 0°C. An equal volume of 0.2 M NaOH, 1% SDS was then added to the mixture, and the incubation was continued for an additional 45 min at 0°C. At this time, 0.2 volumes 3 M NaOAc (pH 4.8) were added. The sample was mixed thoroughly and allowed to sit for 1 hr at 0°C before centrifugation at 20,000 x g (4°C) for 30 min. The supernatant containing the supercropped plasmid DNA was extracted twice with phenol:chloroform (1:1) and once with chloroform:isoamyl alcohol (24:1) and precipitated with ethanol, vacuum-dried and resuspended in 10 mM Tris (pH 7.4), 1 mM EDTA.

Positive Hybridization-Translation Assay

Plasmid DNA from the strongly hybridizing clones screened as described above were identified further by positive hybridization-translation assay according to the technique of Cleveland et al. (1980). Purified linearized plasmid DNA was denatured and bound to a nitrocellulose filter according to the technique of Kafatos et al. (1979). Filters were prehybridized as usual and hybridized for 48 hr at 41°C with unfractioanted poly(A) RNA from cultured human epidermal cells. Specifically hybridized RNA was eluted by boiling and translated in vitro in the presence of [35S]-methionine. Translation products were identified by gel electrophoresis.

Gel Electrophoresis

Electrophoresis of DNA was performed with vertical slab gels cast from 1.2% agarose containing 2.2 M formamide, 20 mM MOPS, 1 mM EDTA, 5 mM sodium acetate (pH 7.0) according to the procedure of Boedtker (1971). These gels were stained with 10 μg/ml ethidium bromide in 0.1 M NaCl for visualization of molecular weight standards.

Electrophoresis of proteins was carried out with 8.5% polyacrylamide (30:0.8 acrylamide:bisacrylamide) gels according to the procedure of Laemmli (1970). All gels were developed by fluorography (Bonner and Laskey, 1974).

Hybridization of 32P-Labeled Probes to Nitrocellulose Blots

DNA in agarose gels was transferred to nitrocellulose paper according to the procedure of Southern (1975). RNA subjected to electrophoresis through formaldehyde agarose gels was transferred to nitrocellulose filter paper according to the procedure of Thomas (1980). After a 24 hr transfer, blots were heated at 80°C for 3 hr under vacuum. Prehybridization in "Seas-a-meal" bag was allowed to proceed overnight at 41°C in 50% deionized formamide, 750 mM NaCl, 75 mM sodium citrate, 20 mM HEPES (pH 7.4), 1 mM EDTA. 100 μg/ml yeast RNA (Miles), 0.01% bovine serum albumin, 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone (PVP), 10 μg/ml sonicated, denatured E. coli DNA and 1 μg/ml sonicated, denatured pBR322 DNA. The prehybridization solution was removed and replaced with a fresh aliquot of the same solution containing 1 x 105 pmol/ml of 32P-labeled cDNA probe (see below). Hybridization was allowed to proceed for 72 hr at 41°C. After hybridization, filters were washed three times for 20 min each in 15 mM NaCl, 1.5 mM sodium citrate and 0.1% SDS at 50°C and then exposed to film.

Preparation of 32P-Labeled CDNA Probes from mRNA and DNA

For use in colony hybridization screening, we prepared 32P-labeled cDNA from mRNA using reverse transcriptase. Typically, a 100 μl reaction contained 0.5 μg mRNA; 50 mM Tris HCl, pH 8.3; 0.5 mM each of dGTP, dATP and dTTP; 10 mM MgCl2; 20 mM dithiothreitol; 70 mM KCI; 10 μM oligo(dT); ... 10 units reverse transcriptase; and 50 μCi 32P-dCTP (400 Ci/m mole, Amersham). The reaction was allowed to proceed for 1 hr at 42°C. EDTA was added to 10 mM and sodium hydroxide was added to 0.15 N and the solution was warmed to 37°C for 10 min. After neutralization, the same was desalted by Sephadex G-75 chromatography.

32P-labeled cloned cDNA sequences were transcribed in vitro essentially as described by Shank et al. (1978). 32P-dCTP was incorporated into short DNA segments by reverse transcriptase, with heat-denatured cloned DNA used as templates. DNA synthesis was initiated with a 1,000-fold weight excess of random oligonucleotide primers derived from calf thymus DNA. Template is rate-limiting in these reactions, and all segments of the DNAs are copied efficiently. The reaction was run for 1 hr at 37°C in the presence of 200 μg/ml boiled pancreatic RNAase A. EDTA was added to 1 mM, SDS to 0.5% and proteinase K (Worthington) to 0.1 mg/ml, and the incubation was continued for 30 min. After extensive extraction with phenol:chloroform (1:1), the aqueous phase was subjected to chromatography through Sephadex G-75 equilibrated in 10 mM Tris·HCl (pH 7.4), 1 mM EDTA and 100 mM NaCl.

Digestion of Genomic DNA with Restriction Endonucleases

DNA samples were digested with either Eco RI, Hind III, Pst I or Bam HI, according to the procedures suggested by New England BioLabs with two exceptions: a fivefold excess of endonuclease was routinely used, and all reactions were allowed to proceed for 4 to 12 hr at 37°C. The extent of digestion was monitored by removing a small aliquot of the reaction mixture and transferring it to a tube containing 1 μg supercoiled pBR322 plasmid. The test samples were incubated in parallel with the main digests. The test samples were analyzed by
Human epidermal DNA was prepared from ten 100 mm dishes of cultured human cells that were grown to three-quarter confluence. Cells on culture dishes were washed thoroughly with 1 mM EDTA (pH 8.0) to remove the fibroblast feeder cells. Cultures were then incubated for 1 hr at 37°C in a solution containing 100 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl (pH 7.4), 0.1% SDS, 2 mM KCl and 100 µg/ml proteinase K (Hughes et al., 1979). The lysed cells were extracted gently but thoroughly with phenol-chloroform (1:1) until the interphase was clear. Three extractions were usually sufficient. The aqueous phase was extracted once with chloroform alone, then dialyzed extensively against 10 mM Tris-HCl (pH 7.4), 1 mM EDTA. Chicken genomic DNA was prepared from 1-day chicken embryos according to the same procedure. DNA from S. purpuratus was prepared from the sperm of a single sea urchin in the same fashion. A second sample of S. purpuratus sperm DNA was donated by R. Sh秭l. Yeast DNA (Saccharomyces cerevisiae) was a gift from H. Klein, and hogfus DNA was provided by S. Chan and D. Steiner. Angler fish, human placental and mouse DNA were prepared from whole tissues and were provided by P. Hobart, R. Picket and G. Bell.

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