The Repetitive Structure of the Profilaggrin Gene as Demonstrated Using Epidermal Profilaggrin cDNA*

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Filaggrin is the histidine-rich basic protein that aggregates keratin filaments in fully differentiated cells of the epidermis. Filaggrin is synthesized in the granular cell layer as a high molecular weight precursor protein (profilaggrin) that consists of multiple repeated copies of filaggrin. DNA clones for rat and mouse epidermal profilaggrin have been constructed from sucrose gradient-enriched RNA in order to study the repetitive structure of profilaggrin. These clones hybridize to high molecular weight epidermal mRNA (23 kilobase pairs, rat and 19 kilobase pairs, mouse) and exhibit limited cross-hybridization between species. Several rat clones directly the synthesis of a portion of rat profilaggrin in bacteria. One of these, rat profilaggrin cDNA clone R4D6, is a 2400 base pairs in length. The R4D6 cDNA is shown to contain repetitive sequence by restriction mapping and southern hybridization analysis of restriction digests of this plasmid, using subfragments of the plasmid as hybridization probes. Southern hybridization analysis of rat genomic DNA, digested to completion with several restriction enzymes, reveals a simple hybridization pattern of fragments equal in size to those of the cDNA. Partial digestion of rat genomic DNA results in a ladder of bands based on a 1200-base pair repeat, equal to the size of the repeating unit of the cDNA clone, and consistent with the expected repeating size of profilaggrin. Together, these results show that the profilaggrin mRNA and gene have repetitive structure and that the gene apparently lacks introns in the coding region.

The epidermis is a complex, developmentally regulated system in which actively proliferating basal cells differentiate stepwise through the spinous and granular cell layer to form cells of the cornified layer (for review, see Odland, 1983). Differentiation is accompanied by changes in cell shape, morphology and the expression of keratin (Fuchs and Green, 1980; Roop et al., 1984; Schweizer et al., 1984). Perhaps the most dramatic change occurs upon the terminal differentiation of the granular cell. At this time major cellular organelles disperse, leaving a cornified cell containing mainly keratin intermediate filaments and filaggrin, within a thickened membrane.

Filaggrin is a histidine-rich protein (Ball et al., 1978; Balmain et al., 1977; Dale, 1977) present in cornified cells which aggregates with keratin filaments in vitro and probably aids in the dense packing of filaments in cornified cells in vivo (Dale et al., 1978; Steinert et al., 1981). Filaggrin is synthesized in the granular cell layer as a large, highly phosphorylated precursor protein (profilaggrin). Profilaggrin, which does not aggregate keratin filaments, is insoluble and accumulates in the keratohyalin granules (Dale and Ling, 1979). During terminal differentiation, profilaggrin is dephosphorylated and proteolyzed at specific sites to liberate mature filaggrin domains which can interact with keratin filaments. Processing signals are presumably present within each linker region.

Our working hypothesis is that profilaggrin (M, in rat >50,000) consists of multiple copies of filaggrin domains interspersed with short linker regions and that these are arranged in tandem (Lonsdale-Eccles et al., 1984; Resing et al., 1984, 1985). This was based on the large size of the mRNA for rat and mouse filaggrin (mouse, 30 S; rat, 34 S) (Meek et al., 1983), and on peptide mapping of profilaggrin that demonstrates a highly repetitive structure (Resing et al., 1984, 1985).

Filaggrin has several unique features. The filaggrins all lack sulfur and are relatively rich in histidine and arginine (Dale, 1977; Steinert et al., 1981). Linker regions of mouse profilaggrin, and probably of other mammalian species, are characterized by the presence of tyrosine-containing peptides which are removed during processing (Resing et al., 1985). Processing signals are presumably present within each linker region.

In order to gain further insight into the structure and function of filaggrin, we have identified several cDNA clones from small libraries prepared from gradient-enriched RNAs. The clones for rat filaggrin are particularly interesting in that they synthesize a filaggrin-related fusion protein. We have used the rat clones to demonstrate that the gene, like the precursor protein, has a highly repetitive structure supporting the tandem-repeat model of profilaggrin structure.

MATERIALS AND METHODS
cDNA Cloning—cDNA was prepared by the method of Gubler and Hoffman (1983) from sucrose gradient-enriched RNA (Meek et al., 1983). Briefly, this method involves RNase H/DNA polymerase I-mediated second strand synthesis, followed by dC-tailing of the double-stranded cDNA and purification on a Sephadex G-50 column equilibrated in TE* + 100 mM NaCl. The plasmid pUC9 (Viera and Messing, 1982) was linearized by digestion with PstI, dG-tailed, and purified as described above. Aliquots of cDNA and vector were mixed in a total volume of 50 μl of TE and annealed at 80 °C for 2 h, followed by slow cooling overnight. The resulting mixes were used to transform Escherichia coli strain RRI, with initial selection for ampicillin resistance.

Oligonucleotide Screening—Colonies were transferred to fresh ampicillin plates in a gridted fashion and incubated overnight at 37 °C.

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‡ The abbreviations used are: TE, 10 mM Tris-HCl, 0.1 mM EDTA (pH 7.5); kb, kilobase; bp, base pairs; SDS, sodium dodecyl sulfate; 50 × Denhardt's, 1% ficoll, 1% polyvinylpyrrolidone.
After transfer to nitrocellulose filters, plasmids were amplified by incubating the filters overnight on nutrient agar plates containing 100 µg/ml of chloramphenicol. This treatment was necessary for optimal signal to noise response of the oligonucleotide probe. Filters were then prepared as described by Grunstein and Hogness (1976), except that colony remnants were removed from the filters by treatment with proteinase K. After washing in 2 X SSC, the filters were incubated in 100 µg/ml proteinase K in 1 X SSC for 30 min until all colony remnants were removed. After drying, the filters were extracted once with chloroform and once in 2 X SSC, 100 µg/ml of chloramphenicol. This treatment was necessary for overnight at 60 °C in 6 X SSC, 10 X Denhardt's, 1% SDS, 0.1% sodium pyrophosphate, 100 µg/ml denatured salmon sperm DNA. Hybridization was carried out at room temperature in the same solution except 100 µg/ml calf liver tRNA was substituted for the DNA. Oligonucleotide probe was added at 10^5 cpm/ml. Filters were washed four times at room temperature in 6 X SSC, 0.1% SDS for 15 min each. Filters were exposed to Kodak X-OMat AR film using an intensifying screen overnight at −20 °C.

The library was screened with an oligonucleotide probe corresponding to a mouse profilaggrin linker sequence (TA C/T TA C/T TAC/T C T GAG/T Tyr-Tyr-Tyr-Glu). The oligonucleotide probe was labeled in a total volume of 10 µl containing 0.2 µg of oligonucleotide, 70 mM Tris-HCl (pH 7.4), 7 mM MgCl2, 1 mM dithiothreitol, 50 µCi [3P] ATP (3000 Ci/mmol), and 10 units of T4 polynucleotide kinase at 37 °C for 60 min. The probe (specific activity 5 x 10^5 cpm/pmol) was then purified on a Sephadex G-50F column which had been pretreated with calf liver tRNA.

Preparation of Nucleic Acids—Plasmid DNA was prepared by a modified alkaline lysis procedure of chloramphenicol-amplified cells (Ish-Horovitz and Burke, 1981). Plasmids were purified on CsCl gradients at 45,000 rpm in a Beckman 70Ti head for 60 min. Colonies were screened for cDNA insert size by the method of Birnboim and Doly (1979).

Epidermis was prepared from newborn C57/B6 mice or Sprague-Dawley rats as previously described (Lonsdale-Eccles et al., 1984). RNA was prepared by the method of Meek et al. (1983). Alternatively, epidermis was homogenized in a fine powder under dry ice, extracted with guanidine thiocyanate, and centrifuged through a CsCl cushion as described (Chirgwin et al., 1979). For genomic DNA, whole liver was ground to a powder over dry ice and suspended in TE containing 2% SDS and 200 µg/ml proteinase K. After incubation overnight at 50 °C, the resulting solution was extracted three times with phenol. DNA was spooled up under 70% ethanol and redissolved in TE for treatment with RNase. The solution was extracted three times with phenol/chloroform, and the DNA collected as above. DNA was dissolved in water for use.

For preparation of specific hybridization probes, pBRD6 (see below) was cut to completion with PstI. Digests (100 µg) were run on long-tough gels (0.7% agarose). Bands were excised and eluted into wells of hydroxyapatite (Tabak and Flavell, 1978). DNA was eluted with 1 M sodium phosphate (pH 7) and desalted on Sephadex G-50F and finally concentrated by ethanol precipitation.

For northern blots (Thomas, 1980), RNA was denatured with glycerol and run on 0.7% agarose gels (Maniatis et al., 1982). For Southern blots (Southern, 1975), genomic DNA was digested with various restriction enzymes and run on 0.7% agarose gels. DNA was transferred to GeneScreen Plus membranes (New England Nucleotids). In 0.4 M NaOH, 0.6 M NaCl. RNA was transferred in 10 X SSC. Blots were prehybridized in 6 X SSC, 10 X Denhardt's, 1% SDS, 0.1% sodium pyrophosphate, and 100 µg/ml denatured salmon sperm DNA overnight at 60 °C. Probe DNA was labeled by the random primer method (Feinberg and Vogelstein, 1983) and purified on a Sephadex G-50F column in TE + 0.1 M NaCl. Hybridization conditions were the same as those used for Northern blots as described above.

DNA Sequence Analysis—DNA sequence was obtained by the dideoxy method using both forward and reverse primers (Sanger et al., 1977). However, as Klenow polymerase could not read through dG tails of the mouse clone, it was necessary to use reverse transcriptase (Smith, 1980). We generally sequence 1 µg of linearized plasmid with 1 unit of reverse transcriptase, followed by electrophoresis on 8% acrylamide gels. Gels were autoradiographed at −20 °C for 3 days. Sequence was analyzed on an IBM PC-XT using the IBI Sequence Analysis Software Package.

Immunofinity Purification of Antibodies—Affinity-purified antibody was prepared as described by Johnson et al. (1986). Briefly, bacterial extract was run on long-tough gels and blotted to nitrocellulose. After incubation with polyclonal antisera, nitrocellulose strips containing bacterial antigen were excised with a razor blade and the antibody eluted as described. Nitrocellulose blots of samples of bacterial extract and rat epidermal extract were incubated overnight with immunoaffinity-purified antibody. Blots were washed and developed using the peroxidase-antiperoxidase complex (Towbin et al., 1979).

RESULTS

Selection of Mouse Profilaggrin cDNA Clone—An oligonucleotide corresponding to linker sequence of mouse profilaggrin was used to screen a cDNA library prepared from high molecular weight mouse epidermal RNA (Meek et al., 1983). Three identical clones were selected. The sequence of one of these clones, designated pM3A10, is presented in Fig. 1.

Limited amino acid sequence data indicate that linkers are somewhat heterogeneous in sequence, although they appear to be similar in overall amino acid composition, being rich in glutamic acid and glutamine, and containing three adjacent tyrosines. The linker sequence derived form the cDNA correlates well with this pattern (Fig. 1, Table I). Linker heterogeneity also explains the presence of a glycine codon in place of a glutamic acid in the fourth position of the probe sequence.

Selection of Rat Profilaggrin cDNA Clones—In order to obtain longer cDNA clones, both the rat and mouse cDNA libraries were screened with the pM3A10 cDNA insert described above. Clones selected from the rat cDNA library were significantly longer. For the 15 cross-hybridizing colonies found to be producing a filaggrin-related protein (Fig. 2) when screened using a monospecific polyclonal antibody against rat filaggrin. The protein pattern is complex, consisting of three main bands at about 85, 70, and 20 kDa. The higher molecular mass peptides are often obtained in somewhat lower yield than the 20-kDa form of the fusion protein. Occasionally, the 85-kDa protein is not detected by immuno blot analysis (see below). One of these clones, designated pBRD6, was chosen for further study. This clone carries a cDNA insert of 2400 bp, a sufficient size to code for a protein of about 90 kDa. Thus, this cDNA has the capacity to code for more than one complete filaggrin domain, plus one or two linker sequences.
sequence of mouse profilaggrin clone M3A10. Purified plasmid DNA was sequenced directly using both forward and reverse primers. Sequence was analyzed using the IBI sequence software package. The correct reading frame was deduced from the reverse primers. Sequence was analyzed using the IBI sequence software.

Fig. 1. Sequence of mouse profilaggrin clone M3A10. Purified plasmid DNA was sequenced directly using both forward and reverse primers. The correct reading frame was deduced from the reading frame of the oligonucleotide probe. Sequences underlined can form stem and loop structures with a serine indicated with an arrow is a potential site of phosphorylation at the 5' end of the cDNA. The presence of a Gly codon in position 4 of the cDNA creates two mismatches with the probe. The G-T pair is allowable and may help to stabilize the A-G mismatch (Szostak et al., 1979). Such mismatches are apparently less destabilizing at the end of hybrids.

Table 1

| Amino Acid | P34' | cDNA | Overall |
|------------|------|------|---------|
| Tyr        | 3    | 3    | 3       |
| Glx        | 13   | 15   | 26      |
| His        | 6    | 2    | 7       |
| Asx        | 1    | 2    | 2       |
| Ser        | 3    | 1    | 3       |
| Pro        | 3    | 1    | 5       |
| Gly        | 1    | 1    | 2       |
| Thr        | 0    | 1    | 1       |

Tryptic peptide derived from a linker region of mouse profilaggrin. Tryptic peptide derived from pM3A10 cDNA. Overall composition of M3A10 cDNA predicted peptide sequence.

Immunological Verification of Rat cDNA Clones—As a means of confirming the identity of the fusion protein and thus the cDNA clone, we used the fusion protein to affinity purify antibody from anti-rat filaggrin serum. Rat filaggrin antiserum reacts with filaggrin, which runs at about 45 kDa on SDS gels, and the high molecular weight profilaggrin precursor which just barely enters the gel. Extracts of pR4D6 bearing cells were prepared, run on gels, and blotted to nitrocellulose. After reacting the blot with antibody, the section of hybrids.

Fig. 2. Immunoblot analysis of rat filaggrin cDNA clones. Extracts of eight bacterial colonies cross-reacting with pM3A10 and a pUC9 control were run on 7.5–15% gradient gels (Laemmli, 1970), blotted to nitrocellulose, and reacted with polyclonal antiserum to rat filaggrin. Background staining is visible in extracts showing a negative immunological response, a pattern similar to that seen using control serum (not shown). The isolates yielding a positive response have cDNAs of identical structure. The three major immunologically reacting peptides are marked with arrows. pR4D6 was chosen for further study.

Fig. 3. Immunoaffinity purification of rat filaggrin antibody. Each panel has samples of epidermal extract (E) and of extracts of bacteria carrying the pR4D6 (B). A, stained gel. B, immunoblot of rat filaggrin antiserum. The higher molecular weight bands of the fusion protein are generally obtained in variable yield. In this experiment, the 20- and 70-kDa bands are visible. In epidermal extracts, a normal immunoblot pattern consists of profilaggrin which barely enters this gel and filaggrin at about 45 kDa plus some degradation products. C, immunoblot using control serum. The staining seen in the bacterial extract lane is background. D, immunoaffinity-purified filaggrin antibody using a nitrocellulose strip containing rat filaggrin as the affinity adsorbant. The eluted antibody yields an identical staining pattern as in B. E, immunoaffinity-purified filaggrin antibody using the 20-kDa form of the fusion protein were excised and used to affinity purify antibody. Specific antibody reaction is identical to that shown in B. Arrows indicate specific antibody reaction with the two fusion protein species present on this blot. The band at around 110 kDa is present in the control serum lane, and thus is nonspecific.

Northern Blot Analysis—The size of profilaggrin mRNA fusion proteins and thus the cDNA clones. It also verifies the specificity of the antiserum and also establishes the relatedness of the high and low molecular weight forms of the fusion protein.
Profilaggrin mRNA is very weak using the short probe, but the cDNA itself carries repetitive sequence (Fig. 4C, lanes 1-3). It shows a smeared hybridization pattern with a maximum size of 19 kb. Liver RNA (Fig. 4B, lane 3), a nonkeratinized tissue which does not synthesize profilaggrin, gives no hybridization signal, indicating that the hybridization patterns are specific.

Profilaggrin Gene Structure—Because profilaggrin protein has a highly repetitive structure, we investigated the possibility of repetitive structure in the R406 cDNA. Preliminary restriction mapping of the R4D6 cDNA insert suggested that the cDNA itself carries repetitive sequence (Fig. 5A), as suggested by two restriction site clusters spaced about 1200 bp apart. The cDNA consists of three PstI fragments in the order 350-1200-850 bp. When the 350-bp PstI fragment was hybridized to PstI digests of the entire cDNA, both the 350- and 1200-bp fragments yielded strong signals (Fig. 5, B and C). Hybridization is localized to the 350-bp fragment and to sequences to the right of the central Bgl II site and to the left of the most central SmaI site.

Further hybridization analysis of R406 cDNA using M3A10 as a probe indicates that a single linker sequence is located within the 1200-bp fragment. Sequence analysis demonstrates that this sequence is highly homologous to M3A10, and likewise contains three adjacent tyrosines (Fig. 5; results to be presented elsewhere). Since M3A10 (mouse linker) does not cross-hybridize with the 350-bp PstI fragment, cross-hybridization between the 350-bp fragment and the 1200-bp PstI fragment must be confined to sequences to the right of the linker region. Thus, repetitive sequences as determined by restriction mapping and hybridization analysis are about 1200 bp apart.

Restriction digests of high molecular weight rat genomic DNA were probed with the 1200-bp PstI fragment on Southern blots in order to investigate rat profilaggrin gene structure. Digestion of rat DNA with PstI yields a 1200-bp fragment

![Fig. 4. Northern analysis of epidermal RNA. A, ethidium bromide-stained gel. Lane 1, λ-DNA standards. Lanes 2-4 are samples of mouse and rat epidermis and rat liver RNA, respectively. Ribosomal RNAs are shown to migrate at about 4718 and 1869 bases for 28s and 18s species, respectively (Chan et al., 1985; Torczynski et al., 1983). B, Northern blot of samples from A: lane 1, mouse epidermal RNA; lane 2, rat epidermal RNA; lane 3, rat liver RNA. Samples were blotted to GeneScreen Plus and probed with labeled 350-bp fragment of R4D6 (see text). The lack of reaction of the probe with liver RNA, indicating specific hybridization. C, cross-hybridization analysis of mouse epidermal RNA with rat profilaggrin cDNA. The probe consisted of the entire R4D6 plasmid. Lanes 1-3 have 2.5, 5, and 7.5 µg of total mouse epidermal RNA and lane 4 has 3 µg of total rat epidermal RNA.](https://example.com/fig4)

![Fig. 5. Repetitive sequence within the R4D6 cDNA. A, restriction map of R4D6 cDNA. Code: P, PstI; B, BamHI; S, SmaI; R, RsaI; SI, SallI; PI, PvuII. PstI and BamHI sites were mapped using partial digestions. Other sites were mapped relative to the single SallI site. The position of the linker sequence is indicated by the thickened line within the 1200-bp PstI fragment. The position of the linker was established by hybridization analysis with M3A10 cDNA and DNA sequence analysis. The arrow indicates direction of transcription. B, stained gel. C, Southern blot of gel in B. PstI digestion was cut with various restriction enzymes. Digests were run on a 0.7% agarose gel and transferred to GeneScreen Plus and probed with labeled 350-bp PstI fragment. Blots were washed at a stringency of 0.2 X SSC at 50 °C. 1, PstI; 2, RsaI; 3, PstI + RsaI; 4, SmaI; 5, PvuII. Note that each restriction digest yields two fragments that hybridize with the 350-bp PstI probe. Cross-hybridization of the 350-bp PstI probe with digests in lanes 2-5 map within the central 1200-bp PstI to the right of the linker and to the left of SmaI site downstream of the linker.](https://example.com/fig5)
which has the same mobility as the large PstI fragment from the cDNA (Fig. 6). Likewise, digestion of high molecular weight rat genomic DNA with BamHI or PvuII gave fragments of identical mobility to fragments of the cDNA. When digests of mouse genomic DNA were probed with the M3A10 cDNA, simple hybridization patterns were also obtained. Digestion with PstI yielded an 850-bp hybridizing fragment (results not shown).

Taken together, these results could be interpreted to indicate that the profilaggrin gene could have a relatively simple repetitive structure. In order to verify this finding, PstI partial digests of high molecular weight genomic DNA were prepared and yielded a ladder of bands that are apparently multiples of the 1200-bp fragment (Fig. 7A). The same hybridization pattern was obtained when either the 350- or 850-bp PstI fragment was used as probe (results not shown). Molecular weight calculations of each fragment of ladder were inconclusive in proving that the ladder of bands seen in Fig. 7A formed a true polymeric series, either because such determinations are normally subject to error, or because of the presence of extra sequences (introns) within the gene not present in the mRNA. To distinguish between these possibilities, the 1200-bp PstI fragment was concatamerized using T4 ligase and blotted in parallel with partial PstI genomic digests. The results of this experiment are shown in Fig. 7B. Each member of the genomic ladder has an identical mobility with each band of the artificially created ladder. Thus, the profilaggrin gene is based on a simple 1200-bp repetitive unit. Furthermore, the coding region of the gene is apparently not interrupted by introns.

**DISCUSSION**

We have used sucrose gradient-enriched RNA from mouse and rat epidermis to prepare cDNA clones for epidermal profilaggrin mRNA. The identity of these clones is established by several criteria. (a) Amino acid composition from the predicted cDNA sequence of the clone for mouse profilaggrin closely matches that of linker peptide sequence derived from mouse profilaggrin protein. (b) The longer rat cDNA clones direct the synthesis of an immunologically detectable filaggrin-related fusion protein in bacteria. (c) The 20-kDa fusion protein can be used to immunoaffinity-purify antibodies which react with rat epidermal filaggrin.

The rat cDNA clone designated R4D6 is 2.4 kb in length, a size sufficient to code for a protein of about 90 kDa. This is consistent with the size of the larger fusion protein produced in bacteria. Expression of rat filaggrin in bacteria yields three major peptides at about 85, 70, and 20 kDa, which are synthesized at low levels. Intracellular proteolysis could explain the observed pattern of multiple bands on immunoblots, although proteolysis must be somewhat site specific since rather distinct bands are obtained. Alternatively, premature termination of transcription or translation could explain these results.

The size of profilaggrin mRNA as determined by Northern blotting is 23 kb for rat and about 19 kb for mouse. These are among the largest mRNAs studied to date. However, rather than appear as a single band, the RNA runs as a smear. This is certainly due in part to degradation of high molecular weight mRNAs during purification and handling, even though the RNA preparations used appear to be largely intact as judged by the integrity of the rRNA markers. This hybridization pattern is specific for keratinized tissue including fore- and hindlimb, while a smeared hybridization pattern appears to be a general property of high molecular weight RNAs and has been observed with rat liver fatty acid synthetase (Nepokroeff et al., 1984), mouse α-spectrin (Cioe and Curtis, 1985), and human apolipoprotein B mRNA (Huang et al., 1985).

When M3A10 is used as a hybridization probe on epidermal RNA, either poly(A)+ enriched or total RNA, a pattern similar to that seen in Fig. 4 is obtained, i.e. weak reaction to mouse epidermal RNA, and strong with rat. Thus, the apparent low degree of cross-hybridization between the rat cDNA clone and mouse profilaggrin mRNA could in part be due to species
divergence, but also could be due to lower profilaggrin mRNA content of mouse epidermis. Clarification of this point will require the ability to accurately quantitate the amount of filaggrin mRNA synthesized by the epidermis.

Analysis of tryptic peptides of filaggrin and profilaggrin indicate that profilaggrin must be highly repetitive in structure (Resing et al., 1986). Our present working model of profilaggrin structure is that about 16 of these repeated units are tandemly arranged and separated by linker peptides. Southern hybridization results directly demonstrate the repeating structure of the profilaggrin gene, based on a 1200-bp unit, and thus confirms the protein model. A minimum of 13 bands could be counted on the original autoradiogram of the partially digested genomic DNA, which are integrals of a 1200-bp unit. Further refinement of this value will require cloning and sequence analysis of the profilaggrin gene.

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