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Absence of a Single Repeat from the Coding Region of the Human Involucrin Gene Leading to RFLP

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Summary

The human involucrin gene has been mapped to the region q21-q22 of chromosome 1. Three of six Utah families examined were polymorphic for a *Pst*I fragment of the involucrin gene. In one individual, the variant *Pst*I fragment was found by DNA sequencing to be missing one of the 39 repeats that make up two-thirds of the coding region.

Introduction

As the keratinocytes of epidermis and other squamous epithelia terminally differentiate, they produce a highly insoluble envelope subjacent to the plasma membrane (Matoltsy and Matoltsy 1966; Sun and Green 1976). Since the proteins of this envelope are cross-linked by a transglutaminase-catalyzed reaction, the envelope is resistant to detergents and reducing reagents (Rice and Green 1977; Sugawara 1979).

The cytosolic protein, involucrin, is an abundant transglutaminase substrate and envelope constituent (Rice and Green 1979; Simon and Green 1984). A large part of the coding region of the human involucrin gene consists of 39 repeats of a sequence of 10 codons (Eckert and Green 1986). A segment of repeats homologous to that of the human is found only in anthropoids (Tseng and Green 1988, 1989; Djian and Green 1989; Teumer and Green 1989). This segment of repeats is therefore called the modern segment. The rest of the coding region is present in lower animals as well (Tseng and Green 1988; Simon and Green 1989).

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2. Owing to his untimely death on August 28, 1988, Dr. Latt did not participate in the writing of the manuscript. This paper is dedicated to his memory.

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Size-polymorphism for involucrin is common in nonhuman primates (Parenteau et al. 1987). This is due to the presence of a variable number of repeats in the modern segment (Teumer and Green 1989; Tseng and Green 1989). In three of six Utah families screened, we found that the RFLP of the human involucrin gene is due to a shortened *Pst*I fragment within the coding region of the gene.

Material and Methods

Somatic Cell Hybrids

The human-rodent somatic cell hybrids were derived from fusions of hypoxanthine phosphoribosyltransferase–deficient Chinese hamster E36 or mouse RAG cells with white blood cells from a normal male or from unrelated female carriers of the X/19 translocations t(X;19)(q24::q13) (Latt et al. 1976) and t(X;19)(q13::p13) (Brook et al. 1984), or with fibroblasts from a female carrier of an X/13 translocation t(X;13)(q22::q22) (Latt et al. 1976). A single hybrid cell line derived from fusion of mouse A9 cells with fibroblasts from a female carrier of the X/11 translocation t(X;11)(q25–26::q23) has been described elsewhere (Scott et al. 1979). Approximately 10 μg DNA from each of the hybrid cell lines, from the rodent parental cells, and from lymphoblastoid cells of a karyotypically normal male were digested with *Hin*dIII. Digested DNAs were separated on a 1% agarose gel in 90 mM Tris (hydroxymethyl) aminomethane-borate buffer, pH 8.3, containing 2.5 mM Na_2 EDTA (TBE buffer). The DNA in the gel was

denatured in 0.5 M NaOH, 0.5 NaCl, neutralized in 0.5 M Tris (hydroxymethyl) amino methane–HCl buffer, pH 7.6, containing 0.6 M NaCl, and transferred to nitrocellulose in 0.3 M sodium citrate, pH 7.0, containing 3.0 M NaCl (20 × SSC). Filters were then incubated for 18 h at 42°C in 0.06 M sodium citrate, 0.05 M sodium phosphate, pH 6.8, containing 0.6 M NaCl, 0.001 M Na_2EDTA, 0.04% BSA, 0.04% ficoll, 0.04% polyvinylpyrrolidone, 0.1% SDS, tRNA at 100 μg/ml, salmon sperm DNA at 25 μg/ml, 8% dextran sulfate, and 50% formamide and then hybridized for 18 h under the same conditions with a 6-kb *Hin*dIII/*Bam*HI fragment derived from pλI-3H6B, containing the entire human involucrin gene (Eckert and Green 1986), labeled with ^{32}P-dCTP by the oligonucleotide priming method (Feinberg and Vogelstein 1983). Filters were washed at 55°C in 0.1 × SSC, 0.1% SDS and exposed to XAR-5 film (Kodak) at −70°C in the presence of an intensifying screen.

In Situ Hybridization

The 6-kb *Hin*dIII/*Bam*HI insert of pλI-3H6B was isolated from a 0.6% low-melting-point agarose gel and labeled with ^{3}H-dATP, ^{3}H-dCTP and ^{3}H-dTTP (Feinberg and Vogelstein 1983) to a specific activity of 5.8 × 10^7 cpm/μg. Preparation of chromosome spreads from phytohemagglutinin-stimulated normal male peripheral blood lymphocytes was carried out according to a method described elsewhere (Bruns et al. 1987). In situ hybridization was performed by the methods of Harper and Saunders (1981) and Harper et al. (1981), as described in detail by Bruns et al. (1987). Slides were R-banded according to the procedure of Donlon et al. (1983) by using chromomycin A3 and distamycin A for probe localization. Some slides were also stained in 0.023 M sodium potassium phosphate buffer, pH 6.8, with 0.06% Wright's stain (Harper et al. 1981; Donlon et al. 1983).

Detection of RFLP in the Involucrin Gene

Lymphoblastoid cell lines derived from Utah kindreds were obtained from the Coriell Institute for Medical Research (Camden, NJ), and their genomic DNA was isolated according to a method described elsewhere (Kunkel et al. 1982). The following kindreds were examined: K1329 (family 981), K1331 (family 982), K1333 (family 983), K1340 (family 984), K1341 (family 985), and K1345 (family 1029).

DNA was digested with restriction enzymes according to the manufacturer's specifications. Digests were electrophoresed through 0.8% or 1.2% agarose gels in TBE buffer, transferred to nylon membranes (Amersham, Arlington Heights, IL; or ICN Biomedicals, Inc., Irvine, CA) in 20 × SSC, and hybridized with a ^{32}P-labeled involucrin probe (pλI-3H6B or a fragment derived from this clone). Filters were washed under stringent conditions (at 56°C in 0.1 × SSC, 0.25% SDS or at 65°C in 0.5 × SSC, 0.17% SDS), and radioactivity was detected on XAR-5 film (Kodak) which was exposed at −70°C with an intensifying screen.

Cloning and Sequencing of the Variant Involucrin Allele

Genomic DNA from a single individual (GM7014) showing RFLP was digested with *Pst*I and electrophoresed through a 1.0% agarose gel, and a 2-mm slice of the gel in the 0.8-kb region was excised (Nicholls et al. 1985). The gel was then dissolved in NaI, and the DNA was absorbed with glass beads (Vogelstein and Gillespie 1979), using a Gene Clean™ kit (Bio 101, Inc., La Jolla, CA). This DNA was ligated into pGem4 (Promega Corp., Madison, WI), digested with *Pst*I, and treated with phosphatase (Maniatis et al. 1982). The ligation reaction was carried out overnight at 15°C, and the DNA was used to transform competent bacteria XL-1 Blue® (Stratagene, La Jolla, CA) or DH5α™ (Bethesda Research Laboratories, Gaithersburg, MD). Plasmid-containing bacteria were selected, using 100 μg ampicillin/ml. The ampicillin-resistant colonies (3,000/150-cm^2 plate) were transferred to nitrocellulose filters (Schleicher and Schuell, Keene, NH) and probed with the 0.85-kb *Pst*I fragment derived from pλI-3H6B, which was labeled with ^{32}P-dCTP. Positive colonies were picked, replated (300 colonies/150-cm^2 plate), and rescreened. Hybridizing colonies were grown in liquid culture, and plasmid DNA was then prepared (Chen and Seeburg 1985) and digested with *Pst*I, and the involucrin fragment was isolated on a low-melt agarose gel. A portion of this DNA was used to generate smaller fragments by further restriction digestion. All fragments were subcloned into M13 for sequencing (Poncz et al. 1982). After transformation into XL-I Blue or DH5α, single plaques were picked and grown for preparation of single-stranded DNA.

Sequencing was carried out by the dideoxy chain-termination method, using Sequenase™ (US Biochemical Corp.; Cleveland, OH) prepared according to the method of Tabor and Richardson (1987). The entire *Pst*I fragment of two independent clones of the variant allele were sequenced from at least one strand; 240 bases encompassing the site of the deletion were sequenced from both strands.

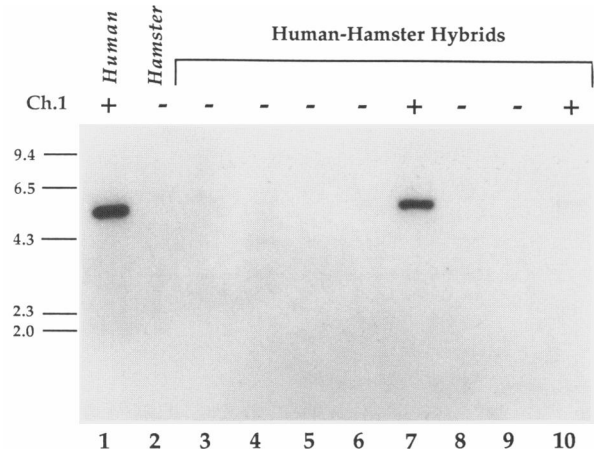

Figure 1 Hybridization pattern of pλI-3H6B with *Hin*dIII-digested DNAs from human-hamster somatic cell hybrids. DNA from human lymphoblastoid cells (lane 1), Chinese hamster E36 cells (lane 2), human-hamster hybrid cells lacking human chromosome 1 (lanes 3–6, 8, and 9), and human-hamster hybrid cells containing human chromosome 1 (lanes 7 and 10) was digested with *Hin*dIII, separated electrophoretically transferred to nitrocellulose, and probed with pλI-3H6B. DNA from those cells containing human chromosome 1 shows hybridization with the probe. The hybrid in lane 10 has chromosome 1 in only a fraction of metaphases and hybridizes only weakly with the probe. The sizes were estimated from those of radiolabeled *Hin*dIII-digested lambda DNA.

Results

Assignment of the Involucrin Gene to Chromosome 1q21-22

For chromosomal assignment, the 6-kb *Hin*dIII/*Bam*HI fragment of the involucrin genomic clone was hybridized with DNAs from a panel of human-rodent somatic cell hybrids. In *Hin*dIII-digested human DNA, a single hybridizing fragment was observed (fig. 1). In the hybrid cell lines examined, this fragment showed complete concordance with the segregation of human chromosome 1 (table 1). The discordant fractions for the other autosomes and the sex chromosomes ranged from .17 to .56. To independently confirm this assignment and to establish the regional localization of the involucrin gene, the 6-kb *Hin*dIII/*Bam*HI fragment was radiolabeled and used for in situ hybridization. As indicated in figure 2, maximal grain density of the involucrin gene probe was observed at 1q21–22. The minor peak at 1p36 could represent a cross-hybridizing species, although Southern blot analysis of DNA from a human-hamster hybrid that contained chromosome 1p but lacked 1q did not reveal a hybridization fragment at reduced stringency. These observations indicate that the involucrin gene can be assigned to chromosome 1q21-q22. (A preliminary account of this work was presented in abstract form by Stroh et al. [1987].)

Table 1

Segregation of Involucrin (pλI-3H6B) with DNAs from Human-Rodent Somatic Cell Hybrids

Chromosome[a]	Hybridization Pattern[b]				Discordant Fraction[c]
	+ / +	− / −	+ / −	− / +	
1	3	14	0	0	.00
2	0	11	2	2	.25
3	3	12	0	3	.17
4	2	11	1	3	.24
5	1	11	2	4	.33
6	2	9	1	5	.35
7	1	10	2	5	.39
8	1	8	2	6	.47
9	1	12	2	3	.28
10	2	9	1	5	.35
11	2	8	1	5	.38
12	2	11	1	3	.24
13	2	9	1	6	.39
14	3	8	0	6	.35
15	1	11	2	4	.33
16	3	10	0	5	.28
17	0	12	3	3	.33
18	0	9	3	6	.50
19 and 19q+[d]	3	5	0	10	.56
20	3	8	0	7	.39
21	3	8	0	7	.39
22	2	12	1	3	.22
X and Xq−[e]	3	8	0	5	.31
Y	0	14	3	0	.18

[a] Human chromosome complements of the hybrids were determined by isozyme and cytogenetic techniques (Bruns et al. 1979) and by analysis of hybrid DNAs with DNA probes for each autosome and the X chromosome.

[b] Column designations are as follows: + / + = involucrin hybridization signal and chromsome both present; − / − = hybridization signal and chromosome both absent; + / − = hybridization present but chromosome absent; and − / + = hybridization absent but chromosome present.

[c] Hybrids with a rearranged chromosome or in which the chromosome was present in fewer than 15% of cells were excluded for calculation of discordant fractions.

[d] For 10 hybrids derived from fusions with leukocytes from the female carrier of the translocation t(X;19)(q24::q13) and for three hybrids derived from fusions with leukocytes from the female carrier of the translocation t(X;19)(q13::p13), this category contains the der19 translocation chromosomes 19pter-q13::Xq24-qter and 19qter-p13::Xq13-qter, respectively.

[e] This category includes hybrids with an intact X and those with derX translocation chromosomes Xpter-q13::19p13qter (one clone) and Xpter-q24::19q13qter (two clones) derived from the above described fusions.

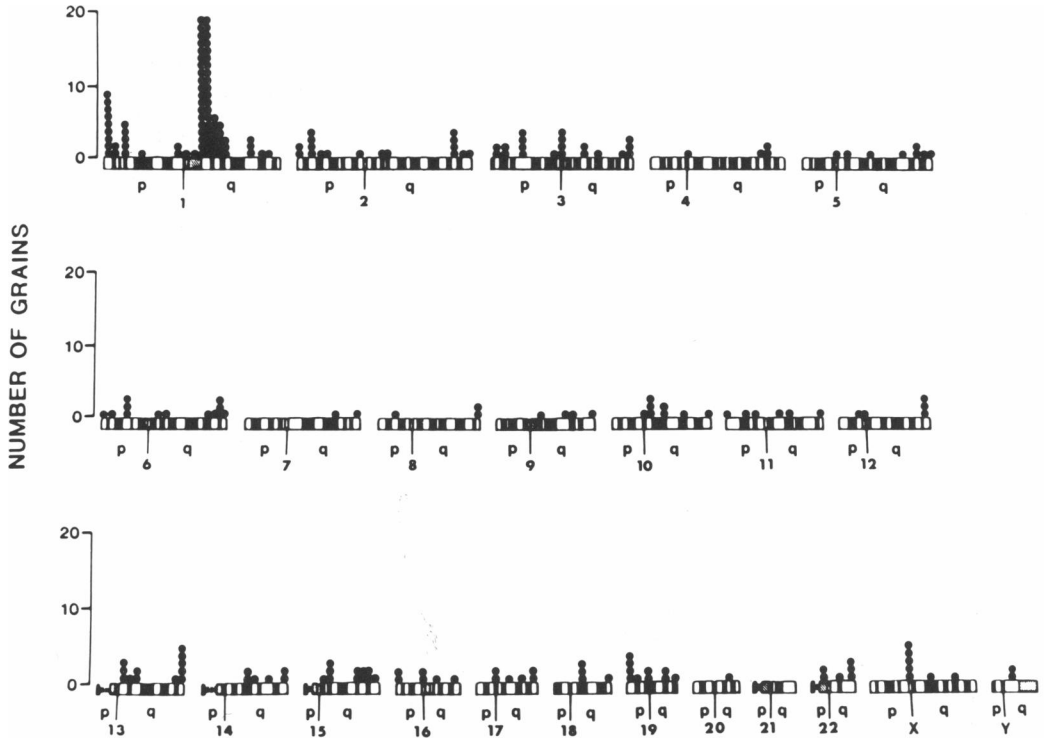

Figure 2 Hybridization of pλI-3H6B to 1q21–q22. This histogram shows the results of the in situ hybridization of pλI-3H6B with human chromosomes isolated from lymphocytes. Hybridization was greatest at band 1q21–q22. Eighty-nine metaphases were scored, and 16% of the 241 silver grains detected localized to 1q21–22. A minor peak of hybridization at 1p36 was also observed.

Short PstI Fragments as a Form of Polymorphism

Initially, DNA samples from the parent pair GM6991 and GM7048 of the Utah kindred K1341 were screened for polymorphism at the involucrin locus. The samples were digested with each of 23 different restriction enzymes and analyzed by Southern blotting. Only digestion with *Pst*I was informative. DNA from GM6991 gave rise to a doublet containing a fragment of 852 bp, as expected from the nucleotide sequence of the coding region (Eckert and Green 1986), and to another fragment slightly smaller. Parent pairs of five additional Utah families were then screened for polymorphism of the *Pst*I fragments. Two of those families (K1329 and K1345) showed similar polymorphism.

The inheritance of the small *Pst*I fragment was tested by following its segregation in two Utah families, kindreds K1345 (fig. 3*A*) and K1329 (fig. 3*B*). In kindred K1345, GM7348 (homozygous for the common large fragment) and GM7349 (heterozygous) produced seven offspring: DNA of four (GM7351, GM7352, GM7354, and GM7356) gave rise to two *Pst*I fragments identical to those of the heterozygous parent and grandparent. In kindred K1329, two heterozygotes, GM7042 and GM7014, produced 10 offspring, of which seven were analyzed. Two (GM6998 and GM7436) showed two *Pst*I fragments similar to those of the heterozygous parents. Three (GM7001, GM7032, and GM7694) were homozygous for the common larger *Pst*I fragment, and two (GM7003 and GM7431) were homozygous for the smaller fragment (fig. 3*B*).

Nucleotide Sequence of the Variant Involucrin Allele of GM7014

The 0.85-kb *Pst*I fragment from GM7349 was cloned, and its nucleotide sequence (figs. 4 and 5) was found to be identical to that described elsewhere (Eckert and Green 1986). The sequence of the 0.82-kb fragment of GM7014 differed from that of the 0.85-kb fragment only by the absence of 10 consecutive codons constituting a typical B^s repeat of the late region: GAG CTC TCT GAG CAG CAG GAG GGG CAG CTG. Confirmation was obtained by sequencing a second clone derived independently from the same genomic DNA.

In order to determine whether the short *Pst*I frag-

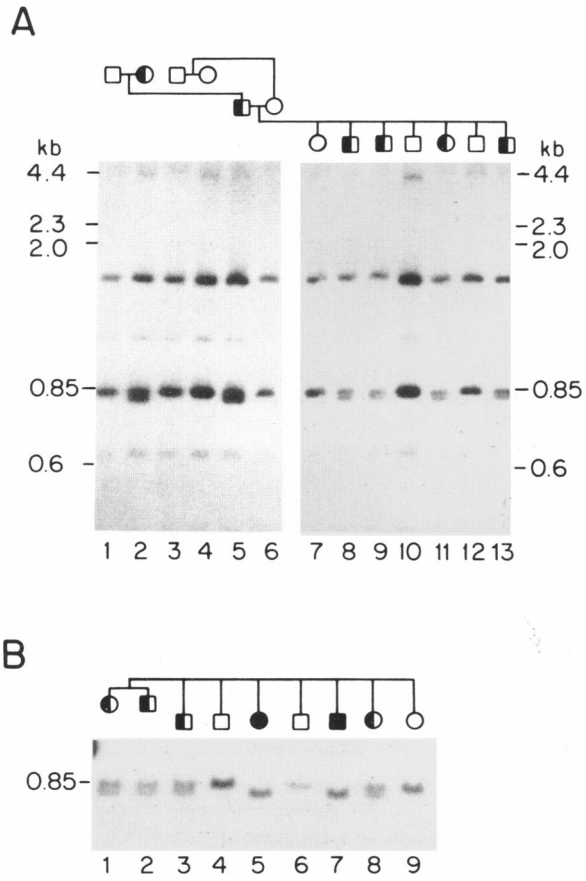

Figure 3 RFLP in two Utah kindreds. A Southern analysis was carried out on *PstI* digests of DNA probed with pλI-3H6B. Only the band at approximately 0.85 kb is informative. The common 852-bp *PstI* fragment is represented by clear symbols. The smaller *PstI* fragment is represented by black symbols. *A,* Utah pedigree K1345. Lanes 1 and 2 show the paternal grandparents GM7347 and GM7346; the former gave rise to a single *PstI* fragment of 852 bp, and the latter produced the same fragment and also the smaller fragment. Lanes 3 and 4 show the maternal grandparents, GM7357 and GM7345, both of which are homozygous for the large fragment. Lanes 5 and 6 show the parent pair, GM7349 (heterozygous) and GM7348. Their seven offspring—GM7350, GM7351, GM7352, GM7353, GM7354, GM7355, and GM7356—are shown in lanes 7–13, respectively; four are heterozygous. *B,* Utah pedigree K1329. Lanes 1 and 2 show the two heterozygous parents, GM7014 and GM7042. Lanes 3–9 show seven of their offspring: GM6998, GM7001, GM7003, GM7032, GM7431, GM7436, and GM7694. Three are homozygous for the large fragment, two are homozygous for the small fragment, and two are heterozygous.

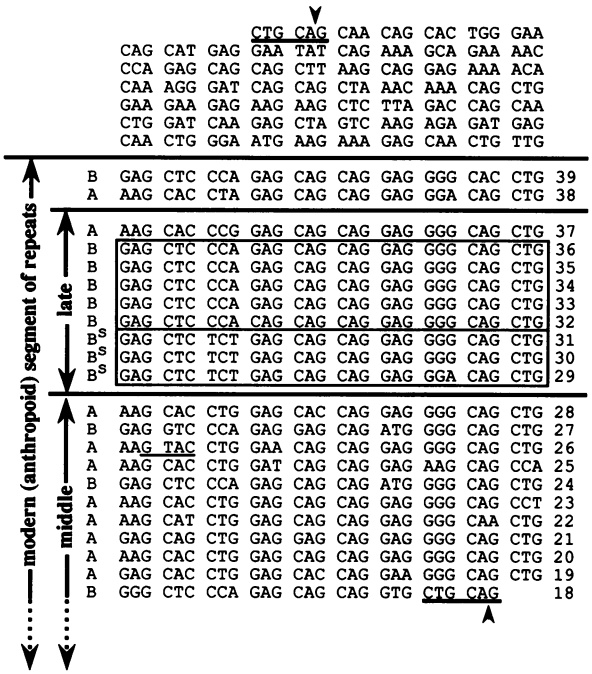

Figure 4 Part of the coding region contained in the 0.85-kb *PstI* fragment. The modern segment of the human involucrin gene contains three types of 10 codon repeats: A, B, and B^S. The consensus sequence of the last seven codons of all three types of repeats is identical. The consensus of the first three codons is as follows: for the A repeat, AAG CAC CTG; for the B repeat, GAG CTC CCA; and for the B^S repeat, GAG CTC TCT. The most common human late region contains five B repeats (32–36) and three B^S repeats (29–31). The large *PstI* fragment contained the sequence shown, including the late region and part of the middle region of the modern segment. This sequence is identical to that published earlier (Eckert and Green 1986), but the repeats are numbered according to the protocol of Teumer and Green (1989). The small *PstI* fragment isolated from GM7014 lacked one of the three B^S repeats in the late region. The *PstI* recognition sites are thickly underlined, and the cutting site is indicated (arrowhead). The *RsaI* site is thinly underlined.

ment in other Utah families might be the same allele as in kindred K1329, we compared the restriction patterns of genomic DNA from all three kindreds cut with *PstI* and *RsaI*, using the 0.85-kb fragment as a probe. For the larger allele, digestion of genomic DNA with *RsaI* and *PstI* yielded two fragments of 590 and 262 bp when probed with the 0.85-kb fragment, and, for the smaller allele, the digestion of genomic DNA yielded fragments of 560 and 262 bp when probed with the 0.85-kb fragment. As expected, the heterozygote GM-7014 of K1329 produced two large fragments of 590 and 560 bp, as well as the fragment of 262 bp.

Genomic DNA of heterozygote members of K1345 (GM7349) and of K1341 (GM6991) gave rise to the same three fragments of 590, 560, and 262 bp. The small *PstI* fragment of these kindreds is therefore likely to be identical to that of K1329.

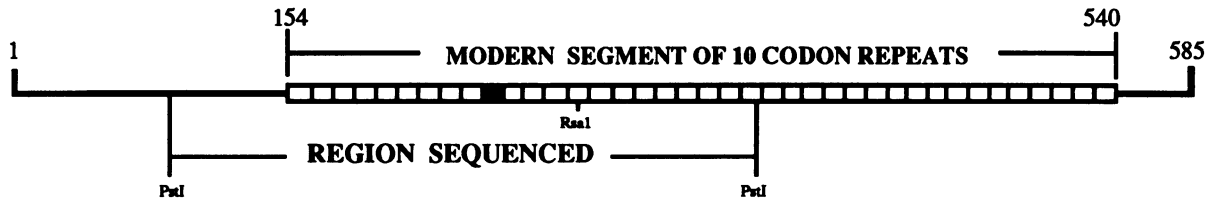

Figure 5 Coding region of the human involucrin gene. The common human involucrin gene contains 585 codons. The coding region contains a segment of 39 repeats of 10 codons (boxed). The 10-codon difference in the small *PstI* fragment of GM7014 is in the segment of repeats and is indicated by the blackened square. Restriction sites are indicated below.

Discussion

During the recent evolution of the involucrin gene of different hominoid species, the modern segment has been extended at or close to its 5′ end by the addition of new repeats (Eckert and Green 1986; Djian and Green 1989; Teumer and Green 1989; Tseng and Green 1989). This late region of the gene is species specific and is a common site of polymorphism in nonhuman primates (Tseng and Green 1988; Teumer and Green 1989), owing to different patterns of repeat addition within the species. The segment of repeats of all anthropoids contains two basic repeats, designated as A and B. The human late region usually contains a block of five B repeats and a block of three B^s repeats (a derivative of a B repeat, so named because it contains a serine codon TCT in the third position). The presence of only two B^s repeats in the small *PstI* fragment could be the result of a deletion, but more likely it is due to failure to generate the third repeat. Since the late region acts as a hot spot for repeat addition, this form of polymorphism would be due to variability in the effectiveness of this hot spot in generating new involucrin-coding sequence in the lineage of these three human families.

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References

Brook JD, Shaw DJ, Meredith L, Bruns GAP, Harper PS (1984) Localization of genetic markers and orientation of the linkage group on chromosome 19. Hum Genet 68:282–285

Bruns GAP, Mintz BJ, Leary AC, Regina VM, Gerald PS (1979) Human lysosomal genes: arylsulfatase A and beta-galactosidase. Biochem Genet 17:1031–1059

Bruns GAP, Stroh H, Veldman GM, Latt SA, Floros J (1987) The 35kd pulmonary surfactant-associated protein is encoded on chromosome 10. Hum Genet 76:58–62

Chen EY, Seeburg PH (1985) Supercoil sequencing: a fast and simple method for sequencing plasmid DNA. DNA 4: 165–170

Djian P, Green H (1989) The involucrin gene of the orangutan: generation of the late region as an evolutionary trend in the hominoids. Mol Biol Evol 6:469–477

Donlon TA, Litt M, Newcom SR, Magenis RE (1983) Localization of the restriction fragment length polymorphism *D14S1* (pAW-101) to chromosome 14q32.1→32.2 by in situ hybridization. Am J Hum Genet 35:1097–1106

Eckert RL, Green H (1986) Structure and evolution of the human involucrin gene. Cell 46:583–589

Feinberg AP, Vogelstein B (1983) A technique for radio-labeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13

Harper ME, Saunders GF (1981) Localization of single copy DNA sequences on G-banded human chromosomes by in situ hybridization. Chromosoma 83:431–439

Harper ME, Ullrich A, Saunders GF (1981) Localization of the human insulin gene to the distal end of the short arm of chromosome 11. Proc Natl Acad Sci USA 78:4458–4460

Kunkel LM, Tantravahi U, Eisenhard M, Latt SA (1982) Regional localization on the human X of DNA segments cloned from flow sorted chromosomes. Nucleic Acids Res 10:1557–1578

Latt SA, Willard HF, Gerald PS (1976) BrdU-33258 Hoechst analysis of DNA replication in human lymphocytes with supernumerary or structurally abnormal X chromosomes. Chromosoma 57:135–153

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Matoltsy AG, Matoltsy MN (1966) The membrane protein of horny cells. J Invest Dermatol 46:127–129

Nicholls RD, Hill AVS, Clegg JB, Higgs DR (1985) Direct cloning of specific genomic DNA sequences in plasmid libraries following fragment enrichment. Nucleic Acids Res 13:7569–7578

Parenteau NL, Eckert RL, Rice RH (1987) Primate involu-

crins: antigenic relatedness and detection of multiple forms. Proc Natl Acad Sci USA 84:7571–7575

Poncz M, Solowiejczyk D, Ballantine M, Schwartz E, Surrey S (1982) "Nonrandom" DNA sequence analysis in bacteriophage M13 by the dideoxy chain-termination method. Proc Natl Acad Sci USA 79:4298–4302

Rice RH, Green H (1977) The cornified envelope of terminally differentiated human epidermal keratinocytes consists of cross-linked protein. Cell 11:417–422

——— (1979) Presence in human epidermal cells of a soluble protein precursor of the cross-linked envelope: activation of the cross-linking by calcium ions. Cell 18:681–694

Scott AF, Phillips JA, Migeon BR (1979) DNA restriction endonuclease analysis for localization of human beta and delta globin genes on chromosome 11. Proc Natl Acad Sci USA 76:4563–4565

Simon M, Green H (1984) Participation of membrane-associated proteins in the formation of the cross-linked envelope of the keratinocyte. Cell 36:827–834

——— (1989) Involucrin in the epidermal cells of subprimates. J Invest Dermatol 92:721–724

Stroh H, Tseng H, Harris P, Bruns GAP, Green H, Latt SA (1987) Chromosomal mapping of the human involucrin gene (IVL). Cytogenet Cell Genet 46:700

Sugawara K (1979) ε(γ-glutamyl) lysine cross-link in the terminal differentiation of mammalian epidermis. Agric Biol Chem 43:2543–2548

Sun TT, Green H (1976) Differentiation of the epidermal keratinocyte in cell culture: formation of the cornified envelope. Cell 9:511–521

Tabor S, Richardson CC (1987) DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Proc Natl Acad Sci USA 84:4767–4771

Teumer J, Green H (1989) Divergent evolution of part of the involucrin gene in the hominoids: unique intragenic duplications in the gorilla and human. Proc Natl Acad Sci USA 86:1283–1286

Tseng H, Green H (1988) Remodeling of the involucrin gene during primate evolution. Cell 54:491–496

——— (1989) The involucrin gene of the owl monkey: origin of the early region. Mol Biol Evol 6:460–468

Vogelstein B, Gillespie D (1979) Preparative and analytical purification of DNA from agarose. Proc Natl Acad Sci USA 76:615–619