Am. J. Hum. Genet. 67:59–66, 2000

Split-Hand/Split-Foot Malformation Is Caused by Mutations in the *p63* Gene on 3q27

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Split-hand/split-foot malformation (SHFM), a limb malformation involving the central rays of the autopod and presenting with syndactyly, median clefts of the hands and feet, and aplasia and/or hypoplasia of the phalanges, metacarpals, and metatarsals, is phenotypically analogous to the naturally occurring murine *Dactylaplasia* mutant (*Dac*). Results of recent studies have shown that, in heterozygous *Dac* embryos, the central segment of the apical ectodermal ridge (AER) degenerates, leaving the anterior and posterior segments intact; this finding suggests that localized failure of ridge maintenance activity is the fundamental developmental defect in *Dac* and, by inference, in SHFM. Results of gene-targeting studies have demonstrated that *p63,* a homologue of the cell-cycle regulator *TP53,* plays a critically important role in regulation of the formation and differentiation of the AER. Two missense mutations, 724A→G, which predicts amino acid substitution K194E, and 982T→C, which predicts amino acid substitution R280C, were identified in exons 5 and 7, respectively, of the *p63* gene in two families with SHFM. Two additional mutations (279R→H and 304R→Q) were identified in families with EEC (ectrodactyly, ectodermal dysplasia, and facial cleft) syndrome. All four mutations are found in exons that fall within the DNA-binding domain of *p63*. The two amino acids mutated in the families with SHFM appear to be primarily involved in maintenance of the overall structure of the domain, in contrast to the *p63* mutations responsible for EEC syndrome, which reside in amino acid residues that directly interact with the DNA.

Introduction

Split-hand/split-foot malformation (SHFM [MIM 183600]) is a limb malformation involving the central rays of the autopod and presenting with syndactyly, median clefts of the hands and feet, and aplasia and/or hypoplasia of the phalanges, metacarpals, and metatarsals (McKusick 1994; Nunes et al. 1995; Crackower et al. 1996; Gurrieri et al. 1996; Raas-Rothchild et al. 1996). There is phenotypic overlap with the EEC (ectrodactyly, ectodermal dysplasia, and facial cleft) syndrome [MIM 129900], in which distal limb malformations sometimes occur as part of the phenotypic spectrum (Roelfsema and Cobben 1996). However, the essential components of the EEC phenotype include not only limb anomalies but also cleft lip and palate with lacrimal duct atresia and defects of the skin, teeth, hair, and nails (Gorlin et al. 1990). Another disorder, Adams-Oliver syndrome (AOS [MIM 100300]) (McKusick 1994) has also been confused with SHFM and EEC. In AOS, variable distal limb reduction abnormalities occur in conjunction with defects of the calvarium and scalp. In addition, ectrodactyly is a major but variable feature of focal dermal hypoplasia (MIM 305600) (McKusick 1994). SHFM is phenotypically analogous to the naturally occurring murine *Dactylaplasia* mutant (*Dac*) (Chai 1981). *Dac* embryos initiate limb development and outgrowth properly, but they fail to form a normal autopod. The results of recent studies have shown that, in heterozygous *Dac* embryos, the apical ectodermal ridge (AER), a critical signaling center that directs the outgrowth and patterning of the developing limb (Saunders 1948), is morphologically normal at E10.5, but, by E11.5, its central segment degenerates, leaving the anterior and posterior segments intact (Crackower et al. 1998). This suggests that localized failure of ridge maintenance activity is the fundamental developmental defect in *Dac* and, by inference, in SHFM (Crackower et al. 1996).

Findings from recent gene-targeting studies have demonstrated that *p63,* a homologue of the cell-cycle regulator *TP53,* plays a critically important role in regulation of the formation and differentiation of the AER. Mice lacking *p63* have striking developmental defects,

Received March 15, 2000; accepted for publication May 8, 2000; electronically published June 5, 2000.

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0002-9297/2000/6701-0010$02.00

including partial or total limb truncations (amelia), abnormal skin, and absence of hair follicles, teeth, and mammary glands (Mills et al. 1999; Yang et al. 1999).

The limb buds of *p63*$^{-/-}$ mice express little or no *Fgf8,* and they lack a morphologically recognizable AER, suggesting that the amelia observed in *p63*$^{-/-}$ mice is the result of failure of AER formation. This indicates that *p63* plays a central role in the initiation of the ridge. Heterozygous mutations in the *p63* gene were recently reported in individuals affected with the EEC syndrome (Celli et al. 1999).

Several reports have described individuals with SHFM who carry specific chromosomal aberrations involving chromosomal regions 7q22.1, 6q16.3-q22.3, 2q24-q31, and Xq24-q27, and these regions have been considered to be candidate areas harboring genes that lead to SHFM (Faiyaz ul Haque 1993; Scherer et al. 1994; Boles et al. 1995; Gurrieri et al. 1995). The results of previous studies in families segregating SHFM as an autosomal dominant trait established the presence of an SHFM locus on 10q24-q25 (Nunes et al. 1995; Gurrieri et al. 1996; Raas-Rothschild et al. 1996; Özen et al. 1999). We have also demonstrated the presence of at least one additional autosomal locus, by the identification of families with a dominant mode of inheritance in whom there was failure to show linkage to 10q, 7q, 6q, or 2q (Gurrieri et al. 1996). To assess the potential of *p63,* which is localized on 3q27, as a candidate gene for SHFM, two multigenerational families in whom segregation analysis had excluded linkage to all previously identified autosomal regions were analyzed for the presence of *p63* mutations (Gurrieri et al. 1996).

Material and Methods

Mutation Identification

Genomic DNA was isolated from peripheral blood samples from family members, according to established protocols. Blood samples were obtained after informed consent was received. The study was approved by the institutional review board of the University of Connecticut Health Center. Intronic primers were used to specifically amplify fragments encompassing each of exons 5–8 from genomic DNA from each of the families. The exon 5 primers, which produced a 284-bp fragment, were TCTCCTTCCTTTCTCCACTGGC (S) and TGCCCACAGAATCTTGACCTTC (AS). The exon 6 primers, which produced a 259-bp fragment, were GCACCAACATCCTGTTCATGC (S) and GTCTACTCAGTCCATAGAGGTGTTG (AS). The exon 7 primers, which produced a 245-bp fragment, were GAAGGAACAACGTCAGTTTAAACCC (S) and AAAGCAGCCACGATTTCACTTTGCC (AS). The exon 8 primers, which produced a 259-bp fragment, were GTGGTAGATCTTCAGGGGACTTTC (S) and CCAACATCAGGAGAAGGATTC (AS). The resulting PCR fragments were screened for the presence of mutations by direct sequence analysis using a PE Cycle Sequencing Kit (PE Biosystems).

Segregation Analysis

For segregation analysis of the K194E mutation by SSCP, the 284-bp PCR fragment encompassing exon 5 was analyzed on native polyacrylamide gels. DNA fragments were mixed with a twofold excess of formamide-dye mix, were denatured for 2 min at 95°C, and were loaded onto a 10% polyacrylamide gel (39:1 acrylamide:*bis*-acrylamide ratio). Gels were run for 8 h at 15 V/cm at 4°C, and fragments were visualized by silver staining.

For segregation analysis of the R280C mutation by restriction-enzyme analysis, the 245-bp fragment encompassing exon 7 was subjected to digestion with the restriction enzyme *Msp*A1-I. PCR product (10 ml) was diluted in 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, 100 mg BSA (pH 7.9) per ml, and 10 U *Msp*A1-I in a total volume of 20 ml. After incubation for 5 h at 37°C, digestion products were analyzed by electrophoresis through a 1.5% agarose gel.

Protein Modeling

A ribbon diagram of the *p63* DNA-binding domain was produced on the basis of the structure of its homologue, *TP53*. Amino acid residues 123–340 of the *p63* gene (GenBank accession number BAA32593), which correspond to the DNA-binding domain of the *TP53* protein (Cho et al. 1994), were folded using a modeling program (Lund et al. 1997). Manual docking of the *p63* structure was performed over the *TP53* DNA-binding-domain structure by use of MOLMOL software (Koradi et al. 1996).

Results

Clinical Phenotype

Both affected families were members of the Cape Province mixed-ancestry population, with genetic endowment from San, Khoi-Khoi, Javanese, indigenous African, and European sources. Members of family R were examined (by P.B.) during ascertainment in 1976. They were reexamined in detail one decade later, and their clinical manifestations were described and depicted (Spranger and Schapera 1988). The spectrum of clinical manifestations was broad, ranging from the presence of a split hand in one individual to bilateral monodactyly and unilateral aplasia of the right lower extremity with a split left foot in another individual. Other family mem-

bers had variably split hands and feet with missing phalanges, metacarpals, and metatarsals, with or without syndactyly and webbing (fig. 1). No family members had any significant abnormalities other than those of the extremities. Individuals in family A were examined (by P.B.) during the initial ascertainment in the 1970s, and thereafter they were examined by clinical geneticists at routine genetic clinics in Cape Province. Further clinical appraisal was undertaken when blood specimens were obtained for molecular studies. In 1999, thorough clinical examinations of eight available family members (individuals II-1, III-7, III-9, IV-3, II-5, III-12, IV-5, and II-7) were performed (by D.B.). The phenotypic expression resembled that of family R and ranged from severe "lobster-claw" malformations of the feet in individual II-7 to minor 3/4 syndactyly of the left foot appearing as the only manifestation in individual III-14 (fig. 2). The daughter of individual III-14 had distal duplications of her thumbs bilaterally, with absence of the second and third phalanges on the right hand and an absent second

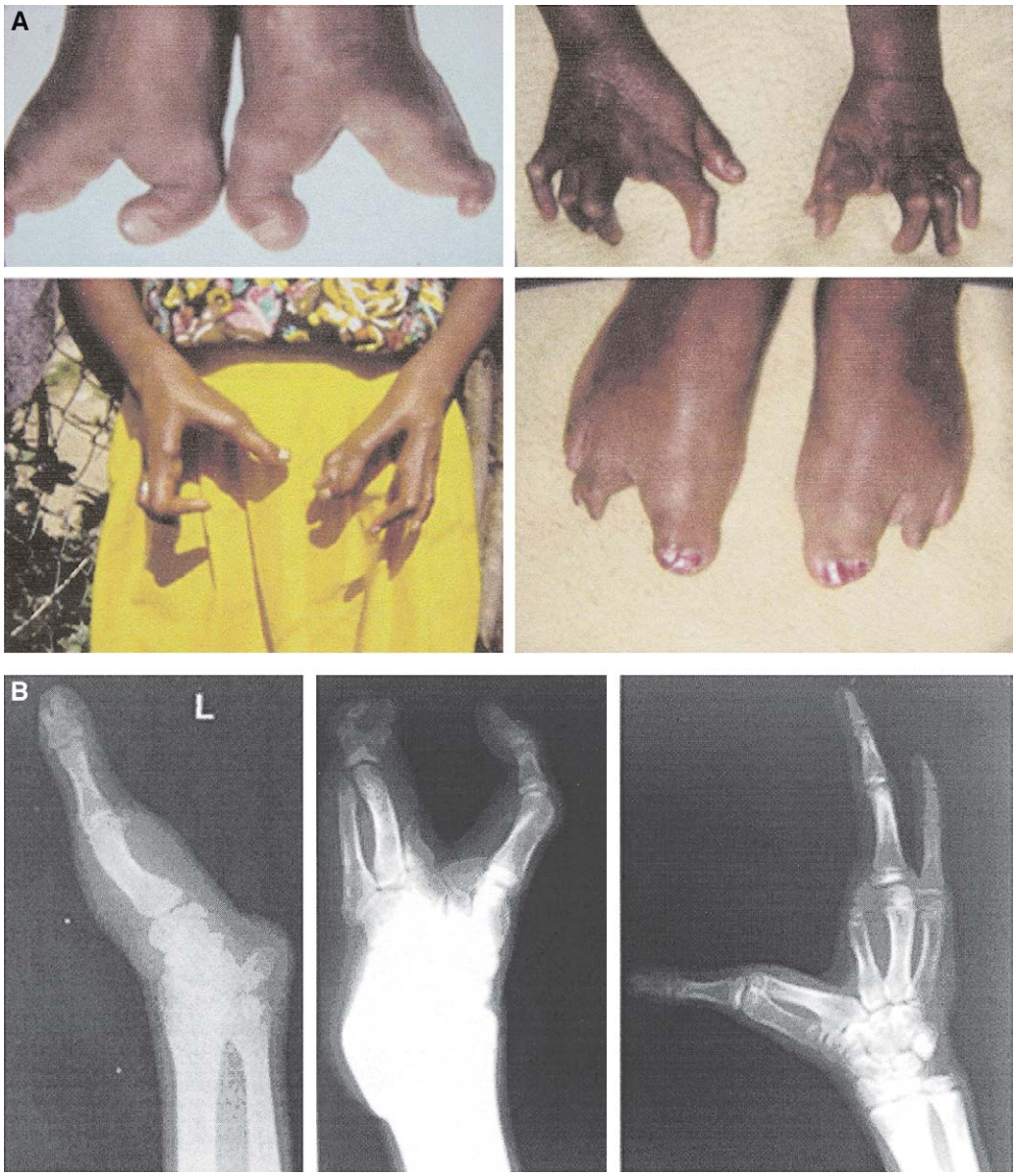

Figure 1 *A*, Family R (*left upper and lower panels*). Note the "lobster-claw" anomaly of the hands and feet. Family A (*right upper and lower panels*). Note the median clefts and syndactyly. *B*, Previously reported (Spranger and Schapera 1988) radiograph of family R (*left*). Note monodactyly with triphalangeal thumb and duplication of the distal phalanx. Family A (*center* and *right*) shows absence of the phalanges of the second and third digits in both foot and hand.

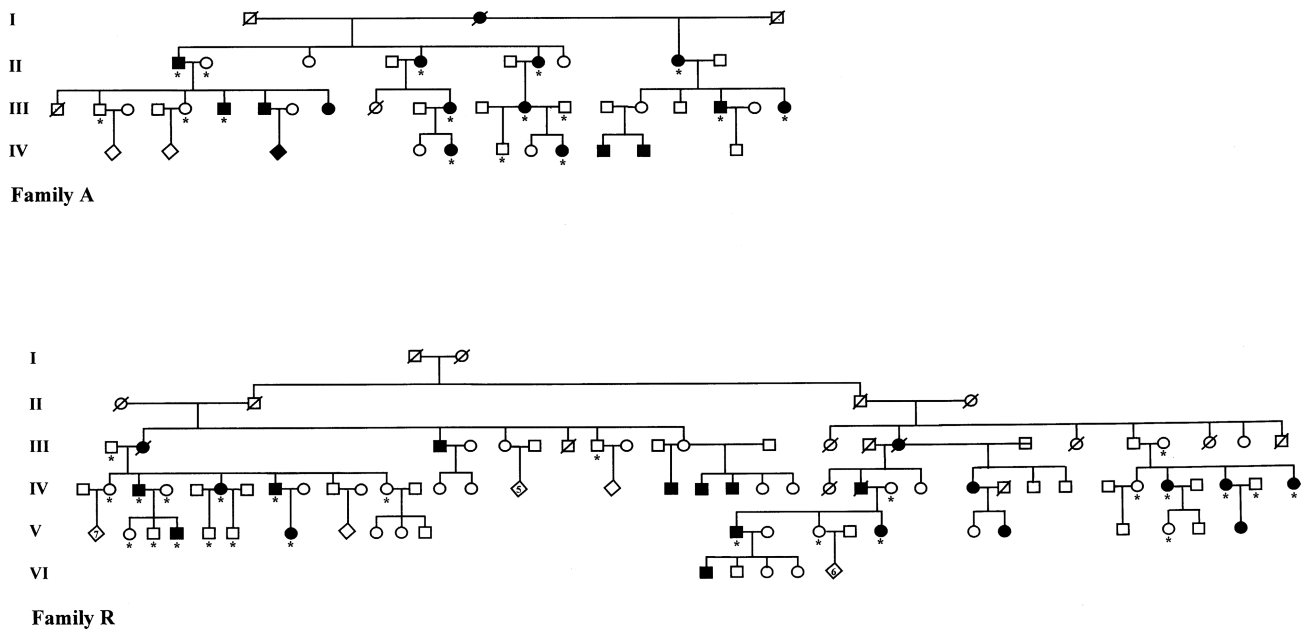

Figure 2 Extended pedigrees of family A (*top*) and family R (*bottom*). Individuals whose DNA was tested are indicated by an asterisk. The phenotypic status of individuals in generations I and II of family R is unknown.

phalanx with 3/4 syndactyly of her left hand. No members of either family had any significant abnormality of the face, palate, skin, teeth, hair, or nails. No abnormalities of the mammary glands or nipples were noted, and the matriarch confirmed that, to her knowledge, none of her family members had any anomalies of this type. Reduced penetrance was deduced in individual III-17 (family A), who was a clinically normal female with an affected parent and two affected offspring (fig. 2). Similarly, in family R, individuals III-11 and III-18, who were phenotypically normal, each had three affected offspring (fig. 2).

p63 *Gene Mutations*

Two missense mutations were identified in exons 5 and 7 in families R and A, respectively. These exons fall within the DNA-binding domain of *p63,* which extends from exons 4–8 (fig. 3*A*). In family R, the point mutation detected by DNA sequencing of two affected individuals was 724A→G, which predicts amino acid substitution K194E (fig. 3*B*). Similarly, in family A, the mutation detected by DNA sequencing of two affected individuals was 982T→C, which predicts amino acid substitution R280C (fig. 3*B*). Neither mutation was detected in a panel of 162 normal chromosomes derived from individuals of diverse ethnic origins, including European, African, and Asian origins. SSCP analysis of DNA from individuals in family R showed cosegregation of the mutation with the SHFM phenotype (fig. 4*A*). Restriction-endonuclease analysis of DNA from members of family A also showed cosegregation of the mutation with the phenotype (fig. 4*B*). Screening of the remaining p63 exons identified no additional sequence alterations in either family A or family R (data not shown). The segregation pattern of the mutations in the two families and their absence from a panel of 81 unrelated normal individuals (data not shown) strongly suggest their causal relationship to the SHFM phenotype.

Mutation screening of the entire p63 coding sequence in four families with the EEC syndrome revealed two additional missense mutations. The clinical phenotype of each family included median clefts of the extremities, cleft palate, and sparse hair without nipple and breast anomalies. All four families with EEC syndrome were of European origin. The mutations were a G→A transition at nucleotide 980 in exon 7 that predicts an arginine-to-histidine substitution at amino acid 279 (R279H) and a G→A transition at nucleotide 1065 in exon 8 that predicts an arginine-to-glutamine substitution at amino acid 304 (R304Q) (data not shown). As was the case for the SHFM *p63* mutations described above, neither the R279H mutation nor the R304Q mutation was detected in a panel of 162 normal chromosomes derived from individuals of diverse ethnic origins.

p63 Protein Modeling

All four of these *p63* mutations are in the DNA-binding domain of the molecule. The possible consequences

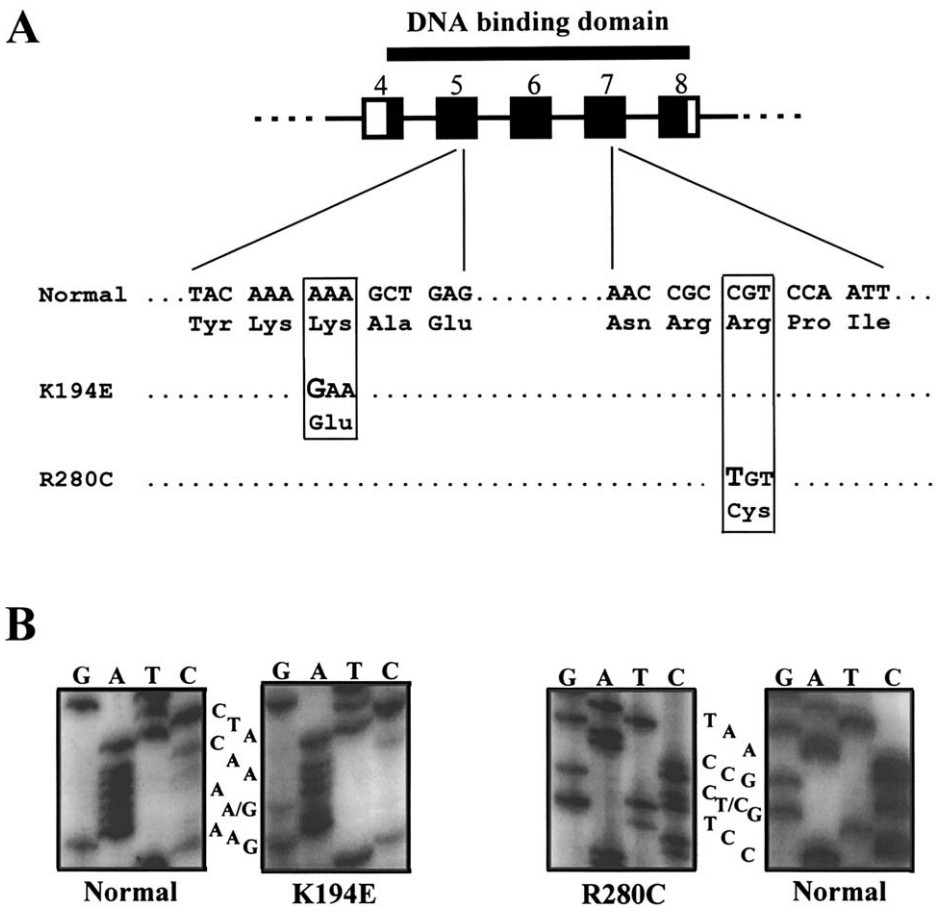

Figure 3 *A,* Schematic representation of exons 4–8 of the *p63* gene, the region that contains the DNA-binding domain. Exons 4–8 were amplified individually from genomic DNA samples, and the resulting fragments were analyzed by means of direct sequence analysis. The K194E and R280C mutations, present in exons 5 and 7, respectively, are shown in comparison with the wild-type sequence. *B,* Identification of the K194E and R280C mutations in individuals with SHFM. The individual with the K194E mutation is heterozygous A/G at nucleotide 724, compared with the normal individual, who is homozygous A at this position. The individual with the R280C mutation is heterozygous T/C at nucleotide 982, compared with the normal individual, who is homozygous C at this position.

of the mutations were assessed by building a model of the DNA-binding domain of the p63 protein on the basis of the structure of its homologue, *TP53,* for which the crystal structure has been solved (Cho et al. 1994). According to this model, it can be deduced that the *p63* DNA-binding domain assumes a loop-sheet-helix motif and two large loops that make up the DNA-binding surface of the protein (Pavletich et al. 1993; Cho et al. 1994) (fig. 5).

Discussion

The p63 amino acids reported herein to be mutated in two families with SHFM are the arginine at position 280 and the lysine at position 194 (fig. 5). The arginine at position 280 is conserved in all three homologues (*TP53, p63,* and *TP73*). This amino acid residue occurs in the L3 loop, and it is surrounded by portions of the L2 and L3 loops and the COOH-terminal end of the S3 strand from the β sandwich. The lysine at position 194 is conserved in *TP73* but not in *TP53*. Remarkably, on the basis of the *p63* model structure, although the arginine at position 280 and the lysine at position 194 are separated by ~100 amino acid residues, these two amino acids reside adjacent to each other in the protein-DNA complex. Rather than directly interacting with the DNA, these two residues primarily appear to be involved in maintenance of the overall structure of the domain. For example, the arginine at position 280 interacts with a number of other amino acids and forms a salt bridge with the carboxylate of the glutamic acid at position 200 (Cho et al. 1994).

In contrast, the two *p63* mutations that we have identified in individuals affected with EEC syndrome reside in amino acids that are directly involved in DNA binding. The arginine at position 279, for example, reaches

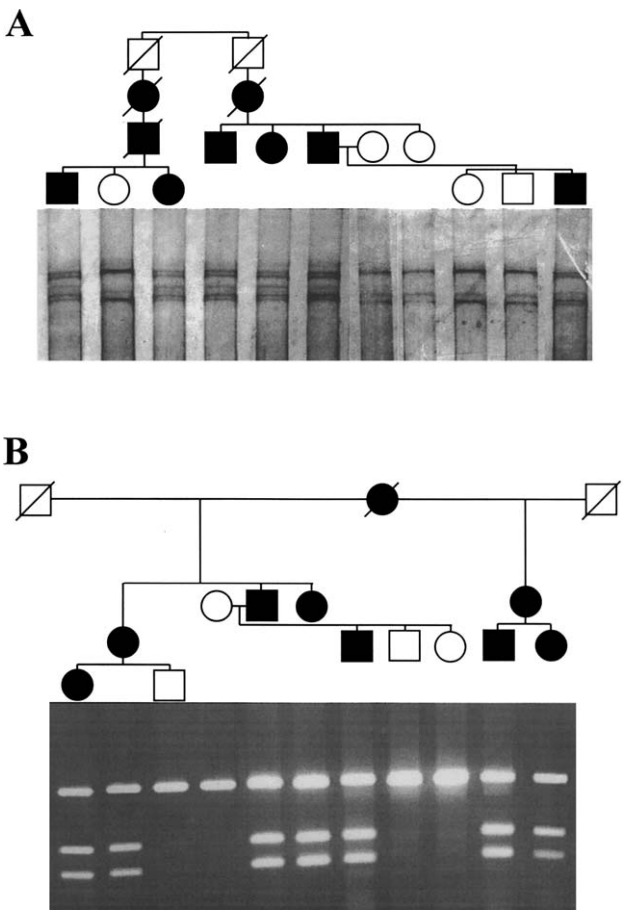

Figure 4 *A,* Segregation of the K194E mutation in family R. The presence of the K194E mutation was detected by SSCP analysis of a 284-bp PCR fragment encompassing exon 5. Normal individuals show two bands corresponding to the two DNA strands, whereas affected individuals show two doublets corresponding to the mutant and normal DNA strands. *B,* Segregation of the R280C mutation in family A. The presence of the R280C mutation was detected by its creation of a CCGCTG recognition site for the restriction enzyme *Msp*A1-I. Digestion of a 245-bp PCR fragment encompassing exon 7 produces subfragments of 138 bp and 107 bp, in addition to the undigested 245-bp fragment, in affected individuals. In normal individuals, only the full-length 245-bp fragment is seen. In both cases, the mutation segregated with the disease in all family members tested. A representative section of each family is shown.

into the minor groove of the DNA, where it interacts with the A-T–rich region of the consensus sequence that is required for specific DNA recognition (Cho et al. 1994). The second amino acid that we have found to be mutated in EEC syndrome, the arginine at position 304, directly contacts a phosphate in the backbone of the DNA. In an earlier study (Celli et al. 1999), mutations in the arginine residue at position 279 as well as mutations of the serine at position 272 have also been identified. The serine at position 272 forms a hydrogen bond with a phosphate in the DNA backbone. Thus, the mutations responsible for EEC syndrome reside in amino acid residues that directly interact with the DNA, in contrast to mutations that we have identified in nonsyndromic SHFM, which reside in amino acid residues that appear to be indirectly involved in DNA binding. It is tempting to speculate that the phenotypic distinction between SHFM and EEC syndrome arises as a consequence of this difference.

The *p63* gene differs from its *TP53* homologue in that it expresses at least six major transcripts (Yang et al. 1998). Three of these transcripts encode proteins with transactivation, DNA-binding, and oligomerization domains similar to those of *TP53,* whereas three lack the N-terminal domain required for transcriptional activation. It has therefore been suggested that *p63* proteins be divided into two classes, one with *TP53*-like properties and the other without *TP53*-associated functions such as transcriptional activation and regulation of apoptosis. Interestingly, it appears that some *p63* isotypes can act as dominant-negative inhibitors of *TP53* and *p63* activation, suggesting a pathogenetic mechanism for the observed heterozygous mutations in patients with SHFM or EEC syndrome (Yang et al. 1998). The apparently opposing properties of the various *p63* isotypes open the possibility of both proliferative and growth-suppressing pathways involving *p63*. It will be of great interest to determine the role of the various *p63* isotypes in the developing limb.

The AER forms in response to signals from limb mesoderm and, in turn, controls the production of a factor in limb mesoderm that feeds back to the AER, maintaining it in an active and functional state. Interference with AER maintenance has been suggested to be the

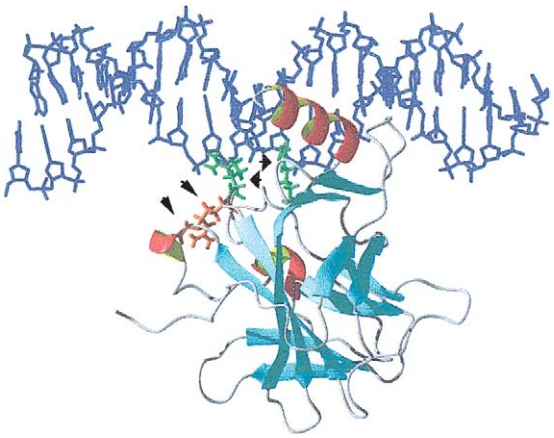

Figure 5 Ribbon diagram of the p63 DNA-binding domain, based on the structure of its homologue, p53. The narrow arrowheads denote side chains of the amino acid residues mutated in SHFM (K194 and R280 [*red*]), and the broad arrowheads denote residues mutated in EEC (R279 and R304 [*green*]).

general pathway affected in SHFM. Thus, the fact that mutations in *p63* result in SHFM suggests that *p63* may also be involved in maintenance of AER activity as well as in formation of the AER. In recent studies of homozygous *Dac* mice (Ianakiev et al. 1999; Sidow et al. 1999), *dactylin,* a member of the WD40/F-box family, has been identified as another molecule involved in maintenance of the AER.

In conclusion, we have demonstrated that mutations in the *p63* gene, a homologue of the archetypal tumor suppressor *TP53* with the potential to be involved in both proliferative and growth-suppressing pathways, cause SHFM. SHFM and the EEC syndrome could be considered to be *p63* morphopathies (Radhakrishna et al. 1999). It remains to be clarified why mutations in the *p63* gene are sometimes associated with nonsyndromic SHFM, as our findings indicate, and, at other times, are linked to EEC syndrome, as reported.

Acknowledgments

The authors wish to acknowledge Dr. Robert Kosher and Caroline Dealy, for helpful comments, and Dr. Assen Marintchev, for help with the molecular modeling. This work was supported in part by a grant from the Coles Family Foundation (to P.T.) and by the University of Cape Town Staff Research Fund and the Mauerberger Foundation (to P.B.).

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/index.html (for the *p63* gene [accession number BAA32593])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for SHFM [MIM 183600], EEC [MIM 129900], AOS [MIM 100300], and focal dermal hypoplasia [MIM 305600])

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