Adult-onset pulmonary fibrosis caused by mutations in telomerase

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Idiopathic pulmonary fibrosis (IPF) is an adult-onset, lethal, scarring lung disease of unknown etiology. Some individuals with IPF have a familial disorder that segregates as a dominant trait with incomplete penetrance. Here we used linkage to map the disease gene in two families to chromosome 5. Sequencing a candidate gene within the interval, *TERT*, revealed a missense mutation and a frameshift mutation that cosegregated with pulmonary disease in the two families. *TERT* encodes telomerase reverse transcriptase, which together with the RNA component of telomerase (*TERC*), is required to maintain telomere integrity. Sequencing the probands of 44 additional unrelated families and 44 sporadic cases of interstitial lung disease revealed five other mutations in *TERT*. A heterozygous mutation in *TERC* also was found in one family. Heterozygous carriers of all of the mutations in *TERT* or *TERC* had shorter telomeres than age-matched family members without the mutations. Thus, mutations in *TERT* or *TERC* that result in telomere shortening over time confer a dramatic increase in susceptibility to adult-onset IPF.

genetics | idiopathic pulmonary fibrosis | telomeres | aging

Idiopathic pulmonary fibrosis (IPF) is a devastating progressive fibrotic disease of the lungs that typically presents after the fifth decade and increases in prevalence with advanced age (1, 2). Mean survival after diagnosis is 3 years (3). The clinical presentation of IPF is similar to that of all of the different scarring lung diseases, collectively called interstitial lung diseases, which lead to pulmonary fibrosis and symptoms of a chronic cough and shortness of breath. IPF is distinguished from the other interstitial lung diseases by its unknown etiology, by characteristic abnormalities on pulmonary function tests and radiographs, and by biopsy findings, which include evidence of injury occurring over time with foci of replicating fibroblasts at the interface between normal and scarred lung tissue (3). A diagnosis of IPF has important prognostic implications. Unlike other interstitial lung diseases, IPF does not respond to immunosuppressive therapies and its clinical course is marked by inexorable deterioration. Currently, no medical therapies have been proven to prolong life expectancy.

Approximately one of every 50 patients with IPF has an affected first-degree family member (4). The inheritance pattern is most consistent with autosomal dominant with incomplete penetrance. The clinical presentation of familial IPF is indistinguishable from sporadic IPF except that the age of onset tends to be earlier (55 years vs. 67 years) (4, 5). Here we report the use of a genetic linkage approach to map the culprit gene in two large families to chromosome 5. Within the linked region we have found multiple mutations in a candidate gene, *TERT*, which encodes the catalytic component of telomerase, and one heterozygous mutation in *TERC*, the essential RNA component of telomerase. Subjects heterozygous for these mutations show evidence of telomere shortening as well as an increased susceptibility to developing IPF.

Results

We have collected 46 families with two or more cases of idiopathic interstitial lung disease, with many of those affected meeting the clinical criteria for IPF (3). To localize the gene defect responsible for IPF, we performed a whole genome, single nucleotide polymorphism (SNP) linkage scan in two of the largest Caucasian families in our collection (Fig. 1). Families F11 and F31 include five individuals with IPF, five with pulmonary fibrosis and six with unclassified pulmonary disease [Table 1 and supporting information (SI) Table 2]. Thorascopic lung biopsies were available from four family members of family F11 (Fig. 2); three biopsy samples had a histologic pattern typical of IPF whereas the fourth (III.4) had generalized fibrosis. Both families displayed linkage to chromosome 5p15 with a maximal logarithm of odds (LOD) score of 2.8. Among the genes we evaluated was *TERT*, which was considered a candidate because a mutation in this gene was recently reported to cause autosomal dominant dyskeratosis congenita (DKC), a disorder in which 20% of affected individuals develop pulmonary fibrosis (6, 7).

The 16 exons and consensus splicing sequences of *TERT* were sequenced for the probands of both families. The proband of family F31 was heterozygous for a deletion of thymidine at position 2240 in the cDNA, which creates a frameshift in the reading frame and is predicted to result in a truncated protein missing half of the reverse-transcriptase domain and the entire C-terminal region. The proband of family F11 was heterozygous for a transition mutation (C*G*T → C*A*T) in codon 865 that is predicted to change a highly conserved arginine to a histidine. This arginine is part of the consensus sequence of motif C, one of seven motifs conserved in all reverse transcriptase proteins (8) (Fig. 3). All family members with IPF or pulmonary fibrosis were heterozygous for these mutations.

For both families, some family members with *TERT* mutations exhibited other clinical features of DKC (7), such as osteoporosis/osteopenia, anemia and cancer (Table 1), but none of the affected individuals had the mucocutaneous lesions typical of DKC. Some family members who inherited the *TERT* mutation had no evidence of pulmonary disease.

We sequenced the coding regions of *TERT* in the probands of 44 additional families with idiopathic interstitial lung disease. Four additional sequence variations, including three missense mutations and one 177-bp deletion, were found (Fig. 3). We also sequenced

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Abbreviations: DKC, dyskeratosis congenita; IPF, idiopathic pulmonary fibrosis; LOD, logarithm of odds; TRAP, telomere repeat amplification protocol; TRF, terminal restriction fragment.

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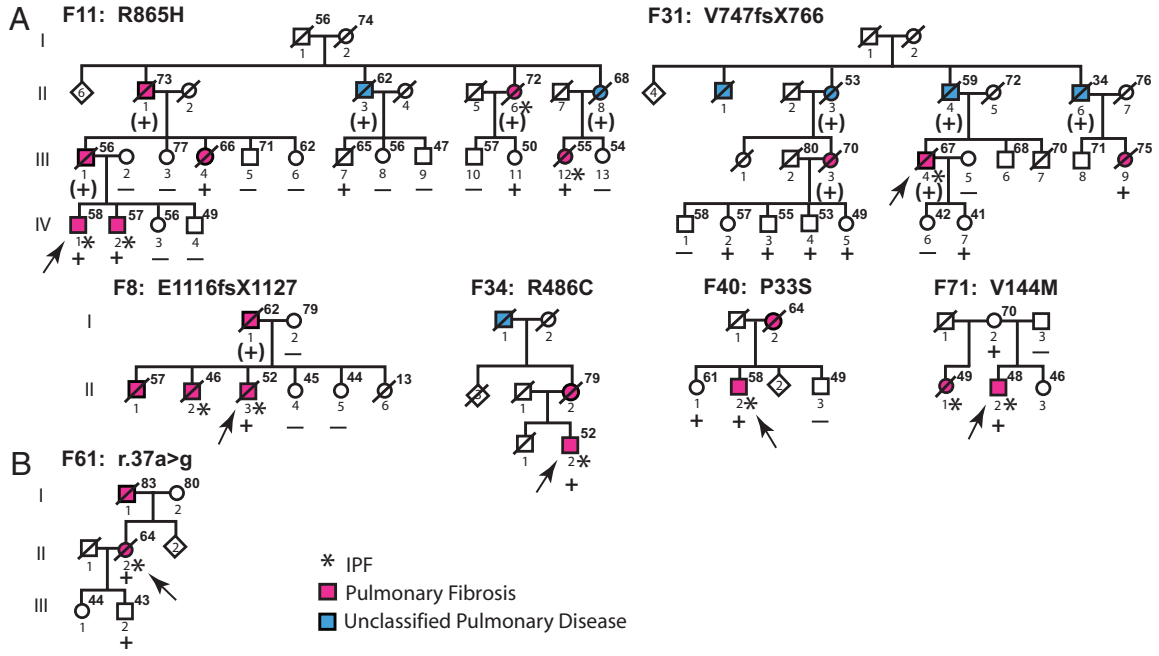

Fig. 1. Abridged pedigrees of seven families with familial idiopathic interstitial lung disease and *TERT* (*A*) or *TERC* (*B*) mutations. Individuals with pulmonary fibrosis or an unclassified pulmonary disease due to a lack of medical studies or records are indicated by red and light blue symbols, respectively. Individuals with IPF are distinguished by the asterisks. The presence or absence of a mutation in either gene is indicated by plus or minus signs, respectively. When the mutation was inferred based on the pattern of inheritance, the plus sign is placed in parentheses. The current age or the age at death is listed to the right of each symbol. The family number and the amino acid change in telomerase reverse transcriptase (*A*) or the mutation in *TERC* (*B*) are listed above each family. Mutations in the DNA and protein sequence are abbreviated by convention (33). Amino acids are listed as single letters.

the coding region of *TERT* in 44 individuals with sporadic interstitial lung disease and no family history of interstitial lung disease. One additional missense mutation was identified involving the same arginine at codon 865 found to be mutated in family F11. Thus, a total of two frameshift deletions and five missense mutations in *TERT* were identified in patients with pulmonary fibrosis (Fig. 3). None of the seven mutations were found in 94 locally collected, ethnically matched individuals, nor were they detected in 200 patients with aplastic anemia or a multiethnic panel of 528 individuals sequenced as controls (9). Electropherograms of all of the sequence mutations are shown in SI Fig. 6, and all common polymorphisms found by sequencing are listed in SI Table 3.

Telomerase has two essential components: the catalytic protein encoded by *TERT* and the telomerase RNA (*TERC*) that provides a template for the repeat sequence added in tandem to the ends of chromosomes. Multiple different mutations in *TERC* have been described for patients with DKC (10). Therefore, we sequenced the 451 bp of genomic DNA that encodes *TERC* in our 46 unrelated probands of familial IPF and in all of the cases of sporadic pulmonary fibrosis. One proband of a Caucasian family (F61) was heterozygous for an adenosine and guanine at position 37. This mutation affects the terminal residue of the P1b helix that is known to function in telomerase template boundary definition (11). This change was not detected in 94 ethnically matched controls but was identified previously in a person with severe aplastic anemia who was a compound heterozygote for variants in *TERC* (12).

To assess the functional significance of the *TERT* and *TERC* mutations, the activity of *in vitro* coexpressed recombinant telomerase protein and RNA was determined by the telomere repeat amplification protocol (TRAP) assay. As expected, the mutation (V747fs, Fig. 4 *A* and *C*) missing half the reverse transcriptase domain and the mutation (E1116fs; Fig. 4*C*) missing the C-terminal E-IV domain had very little detectable telomerase activity. The five missense mutations in *TERT* (Fig. 4 *B* and *C*) and the mutation in *TERC* (Fig. 4 *A* and *C*) produced levels of telomerase activity that ranged from zero to 100% of wild-type activity. Mixing different amounts of the V747fs TERT construct with the wild-type TERT construct did not affect the activity of the wild-type protein, suggesting a mechanism of haploinsufficiency (Fig. 4*D*).

To determine whether the mutations we identified in *TERT* and *TERC* affected telomere length *in vivo*, we analyzed the telomere lengths of genomic DNA isolated from leukocytes. Genomic DNA containing telomeres and subtelomeric regions is resistant to digestion by restriction enzymes and the terminal restriction fragments (TRFs) can be visualized by using concatamers of the telomere sequence (TTAGGG) as a probe using a modification of the Southern blot procedure.

Telomere length is influenced by many different factors, including age (13, 14) and the number of generations the mutation is transmitted (6, 15). When compared with normal family members of similar age, the mean telomere length was significantly shorter for those individuals who were heterozygous for a mutation in *TERT* or *TERC* (Fig. 5 *A* and *B*). All of the mutations caused telomere shortening, even those that were not associated with a detectable decrease in telomerase activity by the *in vitro* TRAP assays. TRFs for all available family members are plotted in Fig. 5*C*. The mean telomere length of asymptomatic heterozygous carriers was similar to those with IPF in the aggregate analysis (Fig. 5*C*).

When telomere lengths were analyzed within families, the family members with clinical disease tended to have shorter TRFs than the asymptomatic carriers. Because the shortest telomeres, not the average telomere length, limit cell growth, (16) we analyzed each family separately and estimated the proportion of short TRFs (arbitrarily set as those between 1.9 and 4.3 kb) for age-similar family members. For family 11 (Fig. 5*D*), the individuals with the highest proportion of short TRFs were the two individuals with IPF. Another individual with a *TERT* mutation in this family had very short TRFs but did not have IPF. This individual had cirrhosis of unknown etiology.

Table 1. Molecular and clinical data of individuals in families with idiopathic interstitial lung disease

Family	DNA change	AA change	Subject	Pulmonary disease: Diagnosis	Smoker	FVC, %	DLco, %	Osteoporosis or Osteopenia	Anemia	Cancer	Other
				TERT mutations							
F8†	c.3346_3522del	E1116fsX1127	I.1	Fibrosis	+				+	Lymphoma	
			II.1	Fibrosis	?						
			II.2	IPF	+	52					
			II.3	IPF	+	62	51				
F11	c.2594G>A	R865H	II.1	Fibrosis	+						
			II.3	Suspected TB	+						
			II.6	IPF	—	51		+	+	Basal cell	
			II.8	Chronic dyspnea	—						
			III.1	Fibrosis	+						
			III.4	Fibrosis	+	43		+‡	+		
			III.7	None	—				+		Cirrhosis
			III.12	IPF	—	42	27			Breast	
			IV.1	IPF	+	106	61	+‡			
			IV.2	IPF	+	70	50	+			
F31	c.2240delT	V747fsX766	II.1	Respiratory failure	?						
			II.3	Suspected TB	?						
			II.4	Emphysema	+						
			II.6	Pneumonia	+						
			III.3	Fibrosis	—	39		+‡	+		
			III.4	IPF	+	72	31			Lymphoma	
			III.9	Fibrosis	—	69*	52	+‡			
F34†	c.1456C>T	R486C	I.1	Suspected TB	?						
			II.2	Fibrosis	?						
			III.2	IPF	+	74	47	+	+		
F40	c.97C>T	P33S	I.2	Fibrosis	+						
			II.1	None	—			+‡			
			II.2	IPF	+	42	31				
F71	c.430G>A	V144M	1.2	None	+			+‡			
			II.1	IPF/Chronic HP	+	56	42				
			II.2	IPF	—	41	25	+	+		
Sporadic	c.2593C>T	R865C		IPF	+	45	33	+			Neutropenia
				TERC mutation							
F61	r.37a>g		I.1	Fibrosis	?						
			II.2	IPF	+	57	14	+‡			
			III.2	Dyspnea and cough	+	69	65				

Pulmonary function test measurements were obtained prior to lung transplantation. FVC, forced vital capacity; DLco, diffusing capacity for carbon monoxide; HP, hypersensitivity pneumonitis; ?, unknown; +, yes; −, no; TB, tuberculosis.
*TLC (total lung capacity) measurement reported.
†All families are Caucasian, except F8 and F34, who are Hispanic.
‡Diagnosis of osteoporosis/osteopenia made prior to or in the absence of treatment with steroids.

Discussion

This report provides previously undescribed molecular insight into the pathogenesis of IPF and expands the scope of diseases caused by mutations in telomerase. The individuals with *TERT* or *TERC* mutations have none of the physical manifestations of DKC and were all ascertained by the lung fibrosis phenotype, distinguishing them from previously described patients with mutations in these genes (6, 9, 10, 17). None had aplastic anemia but several exhibited a mild to moderate anemia and one individual developed neutropenia after lung transplantation. A number of individuals had evidence of axial osteoporosis; in many cases, this diagnosis preceded or was determined in the absence of lung disease. All developed pulmonary fibrosis during their adult years with no history of pediatric lung disease, in contrast with kindreds associated with surfactant protein C mutations (18, 19).

Pulmonary fibrosis appears to be a human-specific manifestation of telomerase deficiency. It is not yet been reported whether aged telomerase-deficient mice also develop similar lung pathology (20). The pathologic features of IPF suggest that the lung injury is focal, affecting scattered portions of the lung parenchyma and recurring over many years (21). Such a pattern of injury could explain why the pulmonary phenotype is incompletely penetrant and may be influenced not only by the nature of the mutations but also by environmental influences, such as cigarette smoking. Smoking causes telomere shortening in a dose-dependent manner (22) and is associated with familial interstitial pneumonia (23). In this study, the average age of death of the smokers with a mutation in *TERT* or *TERC* (58 years, $n = 13$) was 10 years earlier than that of the nonsmokers (68 years, $n = 7$). Telomere length is also influenced by oxidative damage (24) that, in part, may explain why lung disease

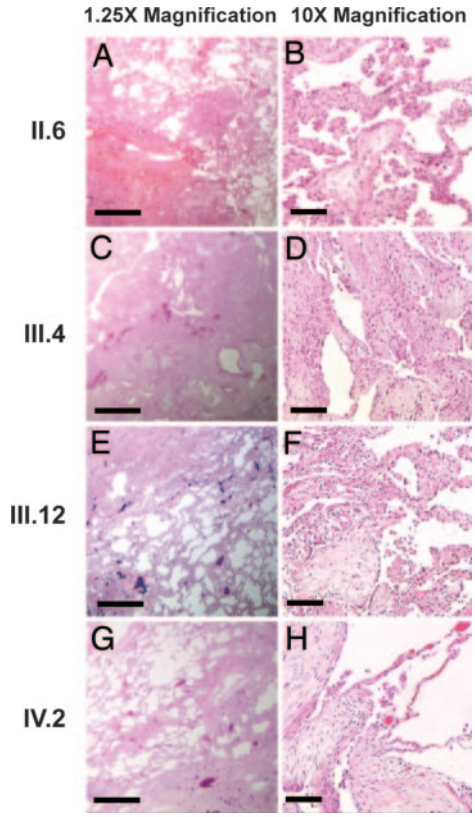

Fig. 2. Histology of lung from thoracopic lung biopsies from individuals II.6 (*A* and *B*), III.4 (*C* and *D*), III.12 (*E* and *F*), and IV.2 (*G* and *H*) from family F11 pictured at ×1.25 magnification (*A*, *C*, *E*, and *G*) and ×10 magnification (*B*, *D*, *F*, and *H*). For individuals II.6, III.12, and IV.2, features of usual interstitial pneumonia are seen with a patchy, heterogeneous pattern of normal lung and densely fibrotic lung tissue with architectural distortion, subpleural and paraseptal fibrosis, honeycombing, thickened alveolar septa, and scattered foci of proliferating fibroblasts. The lung biopsy of III.4 shows the obliteration of most alveoli and their replacement by fibrous tissue, prominent fibroblast proliferation, and an absence of normal pulmonary tissue. (Scale bars: *A*, *C*, *E*, and *G*, 1 mm; *B*, *D*, *F*, and *H*, 100 μm.)

is the predominant phenotype and why smoking may exacerbate this disease. Interestingly, a recent trial with high doses of the antioxidant acetylcysteine appeared to attenuate the progression of IPF (25). Therapeutics directed toward enhancing telomerase activity or delaying telomere shortening may lead to novel treatments for IPF in the future.

Because telomerase protein expression is generally restricted to cells with the capacity to proliferate (26), IPF may result, in part, from the loss or senescence of a cell population in the lung able to respond to repetitive injuries over time. Telomerase may be a marker for identifying resident stem cells that promote regeneration and prevent premature aging of the lung.

Materials and Methods

Clinical Studies. This study was approved by the University of Texas Southwestern Institutional Review Board. Written informed consent was obtained from all participants. Each participant completed a medical questionnaire and medical records were obtained when available. All of the families had two or more cases of idiopathic interstitial lung disease; 34 of the 46 families had individuals with IPF (3). All of the sporadic cases carried a diagnosis of idiopathic interstitial lung disease; 31 individuals had IPF. Genomic DNA was isolated from leukocytes with an Autopure LS (Qiagen, Valencia, CA). For four individuals, DNA was isolated and amplified from formalin-fixed paraffin embedded archival samples as follows. Paraffin was removed from tissue shavings by serial extraction with 1 ml each of xylene, a 1:1 mixture of xylene:ethyl ethanol, and ethyl ethanol. The pellet was dried and the DNA was isolated by using the tissue protocol of the QIAamp DNA Mini Kit (Qiagen). The final product was amplified according to the GenomePlex Whole Genome Amplification Kit (Sigma, St. Louis, MO).

Histology. Photography of hematoxylin and eosin-stained slides of formalin fixed paraffin embedded lung samples were carried out on a Leica DM2000 photomicroscope by using an Optronics DEI-750 analog CCD color camera. Images were captured by using Image J v1.23 acquisition and analysis software (Scion).

Genotyping. Genomic DNA was genotyped by using the Illumina Linkage IVb SNP panel of >6,000 polymorphic SNPs by the University of Texas Southwestern Microarray Core. Call rates varied from 99.7–100% for Autopure-purified DNA and between 72–98% for whole-genome-amplified DNA extracted from archival samples. Individuals with IPF, pulmonary fibrosis, or unclassified pulmonary disease were considered "affected," and all others were assigned an unknown affectation status. We used the software MERLIN (27) to screen the entire genome by using multipoint linkage analysis and a model-free method (28) followed by evaluation of the regions with the highest signals by using a model-based method. Analysis of both families F11 and F31 revealed the highest peak on chromosome 5p15 with a model-free LOD score of 2.82 ($P = 2.0 \times 10^{-4}$) and a model-based LOD score of 2.68 ($P < 2.1 \times 10^{-3}$). The 1-LOD drop interval spanned 4.3 Mb and extended from the end of chromosome 5p to rs959937.

Sequencing and Mutation Analysis. Intronic primers were designed by using ELXR (29) to sequence both exons and splice sites. After purification of the PCR products by using recombinant exonuclease I and shrimp alkaline phosphatase (Exo-Sap, USB), sequencing was performed on an ABI 3700 automated sequencer by BigDye terminator cycle sequencing reagents (Applied Biosystems). All PCR conditions and primers are listed in SI Table 4. All sequences were determined in both directions, the mutations were confirmed by three separate PCR amplification products, and both genes were sequenced in their entirety for all subjects. All sequences used in the comparative alignment were obtained from the NCBI web site at www.ncbi.nlm.nih.gov. The comparison of telomerase reverse transcriptase from different species was based on manual adjustment of a ClustalW generated alignment (www.ebi.ac.uk/clustalw) by using the default settings.

TRAP Assays. Telomerase was reconstituted by expressing human TERT protein and telomerase RNA by using the TnT transcription/translation system (Promega, Madison, WI), and its activity was quantified by the Cy5-fluorescent gel-based TRAP assay as described (30, 31) with slight modification. Mutations were introduced into the parental plasmids pGRN121, pGRN125, and pKT26 (described below) with the use of the QuikChange site-directed mutagenesis kit (Stratagene). The c.2240delT mutation and its wild-type control were generated by PCR and directionally subcloned into pCITE-4a (Novagen). The complete coding sequences for all of the mutants were verified by sequencing. Telomerase RNA was amplified from genomic DNA by PCR using oligonucleotides 5′-GGGGAAGCTTTAATACGACTCACTAT-AGGGTTGCGGAGGGTGGGCCTG-3′ and 5′-CCCCGGATC-CTGCGCATGTGTGAGCCGAGTCC-TGGG-3′, digested with HindIII and BamHI, and subcloned into pUC18 to generate plasmid pKT26. Linearized TERT constructs (0.5 μg) and FspI-linearized telomerase RNA constructs (0.25 μg) were used as substrates in 25-μl TnT reactions. The reactions were serially diluted 1:5 in 1× TRAP buffer starting with 0.3–0.5 μl of the TnT reaction. After the extension of the substrate by telomerase, each sample was treated with 1 unit of RNaseH (Invitrogen)

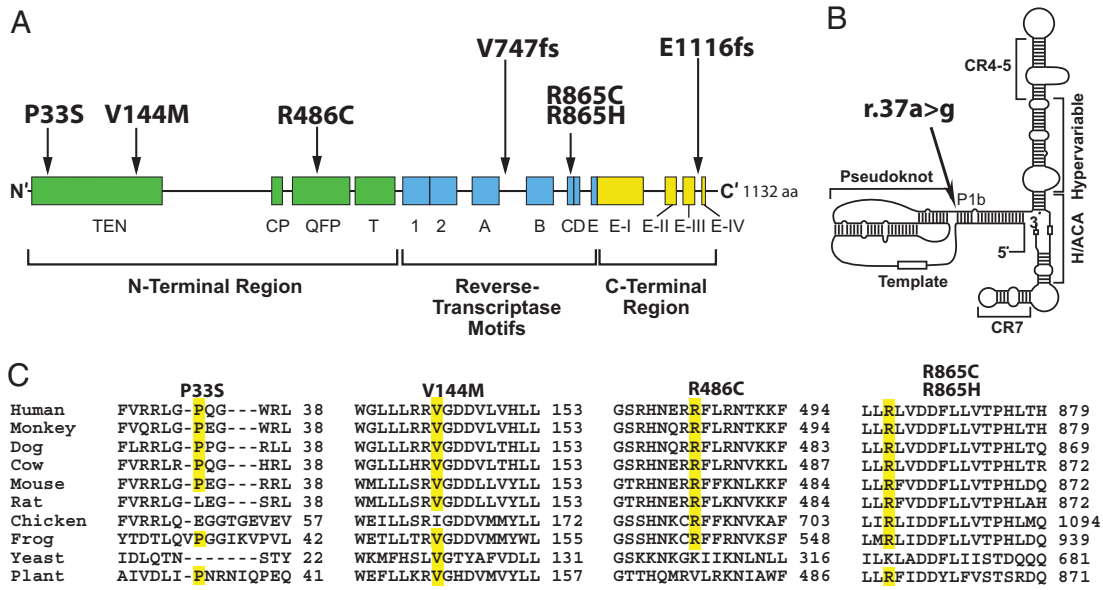

Fig. 3. Schematic representation of the functional domains of telomerase reverse transcriptase (*A*) and telomerase RNA (*B*) with the position of the mutations in IPF patients relative to the domains. (*A*) For the telomerase reverse transcriptase, N-terminal region domains (dark green), reverse-transcriptase motifs (blue), and C-terminal region domains (yellow) are shown. Numbers indicate amino acids. Missense mutations are indicated above the diagram with short arrows; deletions causing frameshifts are indicated by the long arrows. (*B*) Highly conserved domains of telomerase RNA and helices are indicated for telomerase RNA. The r.37a>g mutation disrupts the terminal residue of helix P1b adjacent to the pseudoknot domain. (*C*) Alignment of the telomerase reverse transcriptase sequences of human, *Macaca mulatta* (monkey), *Canis familiaris* (dog), *Bos taurus* (cow), *Mus musculus* (mouse), *Rattus norvegicus* (rat), *Gallus gallus* (chicken), *Xenopus laevis* (frog), *Saccharomyces cerevisiae* (yeast), and *Arabidopsis thaliana* (plant).

for 45 min at 37°C before PCR analysis of the products. Repeat experiments showed the same relative trend of TRAP activities. A total of 0.5 μg of TERT plasmid constructs were used in the mixing experiments. [^{35}S]Methionine-labeled *in vitro* transcribed and translated products were run on a 7% SDS/PAGE gel, fixed in 50% methanol and 10% glacial acid for 1 h, impregnated with Kodak

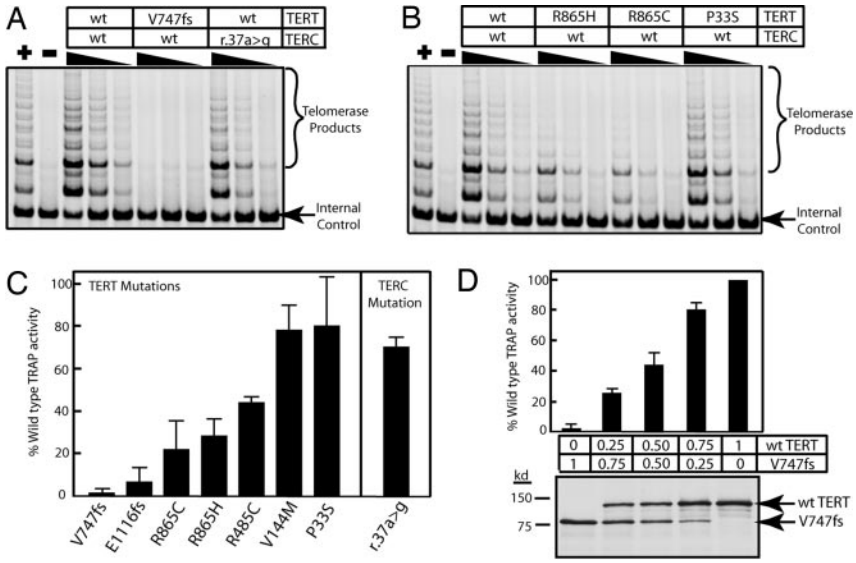

Fig. 4. Telomerase activity of TERT mutants as measured by the TRAP assay. Telomerase activity of *in vitro* coexpressed mutant or wild type (wt) telomerase reverse transcriptase (TERT) proteins with mutant or wild-type telomerase RNA (TERC) (*A* and *B*) were determined by TRAP. Plasmid constructs encoding TERT and TERC were combined as indicated, *in vitro* transcribed and translated together, and serially diluted 1:5 before measuring TRAP activity. The positive control (+) is 250 cell equivalents of H1299, a human cancer cell line known to be positive for telomerase activity, as evidenced by the 6-bp incremental TRAP ladder. An aliquot of the highest concentration of the *in vitro* expressed wild-type telomerase was heat-inactivated at 85°C for 10 min before measuring TRAP activity as a negative control (−). (*C*) Relative amounts of telomerase activity (percent of wild-type TRAP activity) for seven different TERT mutants and one mutation in telomerase RNA were calculated as a ratio of the intensity of the sample's telomerase products to that of the internal control band as described (30) and normalized to wild-type activity in one representative experiment. Error bars represent SD. Parallel TnT reactions were run by using [^{35}S]methionine and run on an SDS/PAGE gel to confirm equal expression of the TERT wild-type and mutant proteins (data not shown). (*D*) Plasmid constructs encoding wt TERT or the V747fs TERT mutant were combined at the indicated ratios, *in vitro* transcribed and translated with TERC, and telomerase activity was measured by TRAP. Parallel TnT reactions were run by using [^{35}S]methionine and run on a SDS/PAGE as shown.

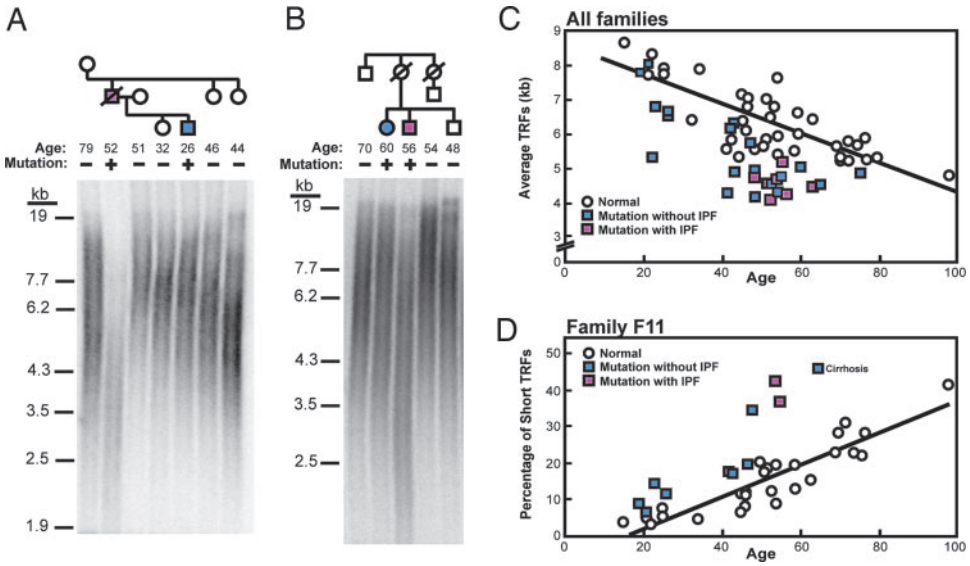

Fig. 5. Telomere length determined by Southern blotting of chromosomal TRFs of genomic DNA isolated from leukocytes of individuals from families F8 (*A*), F40 (*B*), all families (*C*), and family F11 (*D*). Abridged pedigrees, the age of each individual, and the presence (+) or absence (−) of a *TERT* mutation is indicated above each Southern blot in *A* and *B*. Open symbols represent normal individuals, blue symbols indicate individuals heterozygous for a mutation in *TERT* or *TERC* without IPF, and pink symbols represent individuals heterozygous for a mutation with IPF. (*C*) Average TRFs of each individual in all families is plotted against age. (*D*) The percentage of short TRFs (or the relative intensity of TRFs between 1.9 and 4.3 kb over the intensity of TRFs >1.9 kb in length) was plotted against age for all individuals in family F11. Linear regression was used to draw a best fit line through the normal samples.

En^{3}hance (PerkinElmer), dried, and exposed to film at −80°C for 4–16 h.

TRF Analysis. TRF analysis of genomic DNA isolated from leukocytes was performed as described (32). The mean TRF was determined as described except a grid of 200, instead of 30, boxes was placed over each lane. The percentage of short telomeres was calculated by dividing the relative signal intensity of each lane (between 1.9 and 4.3 kb) by the relative signal intensity of the entire lane (between 1.9 and 19 kb).

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