

mine. A single dose of clozapine increases dopamine release in the primate prefrontal cortex, and long-term administration increases basal extracellular dopamine concentration in the prefrontal cortex (21). Although this may not be the only mechanism by which clozapine elicits its effects on PCP-induced cognitive dysfunction, this activation of the dopamine system of the prefrontal cortex may contribute to the ability of clozapine to ameliorate the impairments in our model and, perhaps, in schizophrenia.

Our data show that repeated administration of PCP inhibits basal and stimulated dopaminergic function in the prefrontal cortex of the monkey brain. The deficiency of dopamine in the prefrontal cortex that is induced by repeated administration of PCP is associated with a long-lasting cognitive deficit, which is ameliorated by the atypical therapeutic drug clozapine. These effects are observed long after PCP administration is stopped and thus cannot be attributed to direct effects of the drug. This primate model of dopamine dysfunction in the cortex may provide a paradigm for investigating the pathophysiology underlying neuropsychiatric disorders associated with a primary cognitive dysfunction in the cortex and a dopaminergic deficit in the prefrontal cortex, as is hypothesized in schizophrenia (22). It also may provide a means for evaluating therapeutic agents that are selectively targeted toward alleviating cortical dopamine hypofunction.

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Telomerase Catalytic Subunit Homologs from Fission Yeast and Human

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Catalytic protein subunits of telomerase from the ciliate *Euplotes aediculatus* and the yeast *Saccharomyces cerevisiae* contain reverse transcriptase motifs. Here the homologous genes from the fission yeast *Schizosaccharomyces pombe* and human are identified. Disruption of the *S. pombe* gene resulted in telomere shortening and senescence, and expression of mRNA from the human gene correlated with telomerase activity in cell lines. Sequence comparisons placed the telomerase proteins in the reverse transcriptase family but revealed hallmarks that distinguish them from retroviral and retrotransposon relatives. Thus, the proposed telomerase catalytic subunits are phylogenetically conserved and represent a deep branch in the evolution of reverse transcriptases.

Telomerase is a ribonucleoprotein enzyme responsible in most eukaryotes for the complete replication of chromosome ends, or telomeres (1). Its RNA subunit provides the template for addition of short sequence repeats [typically 6 to 26 nucleotides (nts) to the chromosome 3' end (2). In ciliated protozoa and yeast, telomerase is regulated and the average telomere length is maintained (3). In most human somatic cells, however, telomerase activity cannot be detected and telomeres shorten with successive cell divisions (4). Telomerase activity

reappears in immortalized cell lines and in about 85% of human tumors, which has led to studies of the usefulness of telomerase for cancer diagnostics and therapeutics (5, 6).

Telomerase RNA subunits have been identified and analyzed in ciliates, yeast, and mammals (2, 7), but the protein subunits have been elusive. In *Tetrahymena*, two telomerase-associated proteins (p80, p95) have been described (8), and p80 homologs have been found in humans and rodents (9); the presence of catalytic active site residues in these proteins has not been

established. Purification of telomerase from the ciliate *Euplotes aediculatus* yielded two proteins, p123 and p43 (10), that appear unrelated to p80 and p95 (11). p123 contains reverse transcriptase (RT) motifs and is homologous to yeast Est2 (Ever shorter telomeres) protein (11), which is essential for telomere maintenance in vivo (12). The RT motifs of Est2p are essential for telomeric DNA synthesis in vivo and in vitro (11, 13), supporting the conclusion that Est2p and p123 are the catalytic subunits of telomerase. The question remained whether there are two telomerases in biology, one based on p80- and p95-like proteins and one on p123/Est2p-like proteins.

To determine if Est2p/p123 is conserved among eukaryotes, we searched for homologs in the fission yeast *S. pombe* and humans. Polymerase chain reaction (PCR) amplification of *S. pombe* DNA was carried out with degenerate-sequence primers designed from the *Euplotes* p123 RT motifs B' and C. Of the four prominent products generated, the ~120-base pair (bp) band encoded a peptide sequence homologous to p123 and Est2p. Using this PCR product as a probe for colony hybridization, we identified two overlapping clones from a genomic library and three from a cDNA library (14). None of the three cDNA clones was full length, so we used RT-PCR to obtain the NH₂-terminal sequences (15). This putative telomerase reverse transcriptase gene, *trt1*⁺, encoded a basic protein with a predicted molecular mass of 116 kilodaltons (kD) (Fig. 1A). The sequence similarity to p123 and Est2p was especially high in the seven RT motifs (Table 1) and in motif T (Telomerase-specific) (Fig. 2). Fifteen introns, ranging from 36 to 71 bp, interrupted the coding sequence. All had consensus splice and branch site sequences (16).

If *trt1*⁺ encodes the telomerase catalytic subunit in *S. pombe*, deletion of the gene would be expected to result in telomere shortening and perhaps cellular senescence as seen with the *est2* mutants in *S. cerevisiae* (11, 13). To test this, we created two deletion constructs (Fig. 1A), one removing motifs B' through E in the RT domain, and the second deleting 99% of the open reading frame (ORF). Haploid cells grown from both types of spores showed progressive telomere shortening to the point where hybridization to telomeric repeats became al-

most undetectable (Fig. 1B). Senescence was indicated by (i) reduced ability of the cells to grow on agar, typically by the fourth streak-out after germination; (ii) the appearance of colonies with increasingly ragged edges (Fig. 1C); and (iii) the increasing fraction of elongated cells (Fig. 1D). When individual enlarged cells were separated on the dissecting microscope, the majority underwent no further division. The same *trt1*⁻ cell population always contained normal-size cells that continued to divide but frequently produced nondividing prog-

eny. The telomerase-negative survivors may use a recombinational mode of telomere maintenance as documented in budding yeast strains with deletions of telomere-replication genes (12, 17).

A candidate human p123/Est2p/Trt1p homolog was identified by a BLAST search of the EST (expressed sequence tag) database (GenBank AA281296). This EST was the top-ranked match in sequence searches with *Euplotes* p123 ($P = 3.3 \times 10^{-6}$) and *S. pombe* Trt1p ($P = 9.7 \times 10^{-7}$). The human EST was not found in searches with yeast

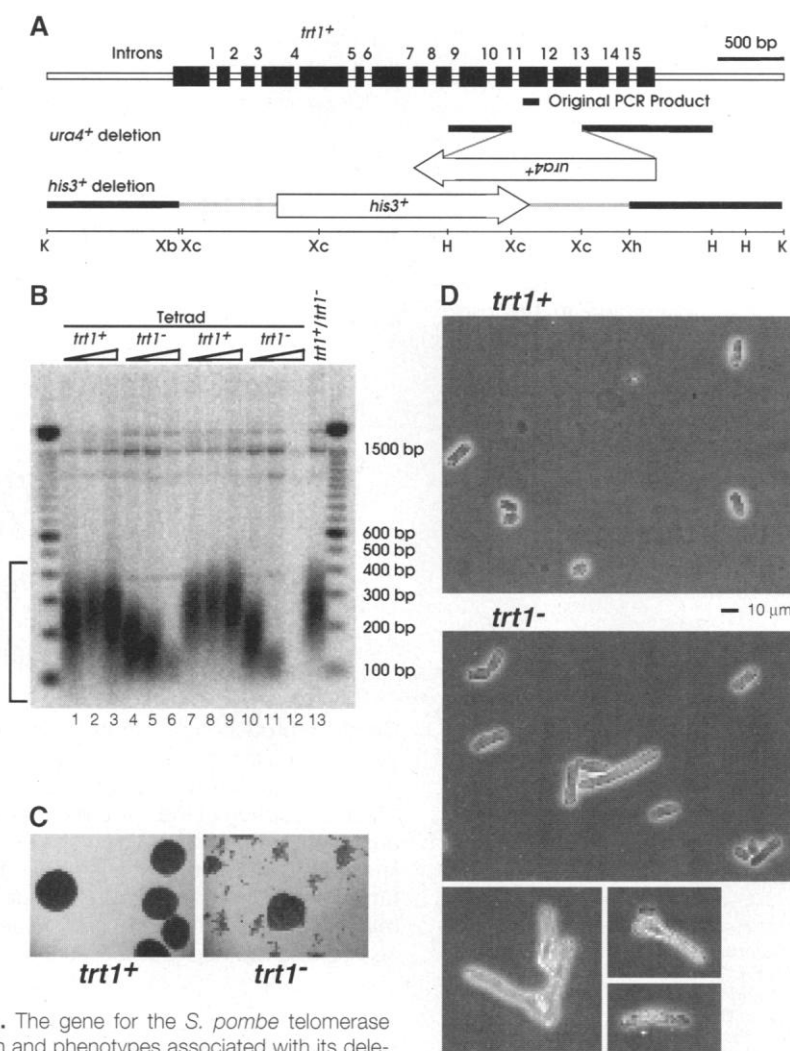


Fig. 1. The gene for the *S. pombe* telomerase protein and phenotypes associated with its deletion. (A) The *trt1*⁺ locus, the location of the ~120 bp PCR product that led to its identification, and the regions replaced by *ura4*⁺ or *his3*⁺ genes in the *trt1*⁻ mutants (K, Kpn I; Xb, Xba I; H, Hind III; Xc, Xca I; Xh, Xho I). (B) Telomere shortening phenotype of *trt1*⁻ mutants. A *trt1*⁺/*trt1*⁻ diploid (28) was sporulated and the resulting tetrads were dissected and germinated on a YES (Yeast Extract medium Supplemented with amino acids) plate (29). Colonies derived from each spore were grown at 32°C for 3 days, and streaked successively to fresh YES plates every 3 days. A colony from each round was placed in 6 ml of YES liquid culture at 32°C and grown to stationary phase, and genomic DNA was prepared. After digestion with Apa I, DNA was subjected to electrophoresis on a 2.3% agarose gel, stained with ethidium bromide to confirm approximately equal loading in each lane, transferred to a nylon membrane, and hybridized to a telomeric DNA probe. The Apa I site is located 30 to 40 bp away from telomeric repeat sequences in chromosomes I and II. (C) Colony morphology of *trt1*⁺ and *trt1*⁻ cells. Cells plated on MM [Minimal Medium (29) with glutamic acid substituted for NH₄Cl] were grown for 2 days at 32°C prior to photography. (D) Micrographs of *trt1*⁺ and *trt1*⁻ cells grown as in (C).

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Est2p, but subsequent pairwise comparison of these sequences showed a convincing match. Sequencing of the rest of the cDNA clone containing the EST revealed all eight TRT (Telomerase Reverse Transcriptase) motifs, but not in a single ORF (18). We used the sequence information from this incomplete cDNA clone to isolate an extended cDNA clone from a library of 293 cells, an adenovirus E1-transformed human embryonic kidney cell line (19). This cDNA clone (pGRN121) had a 182-bp insert relative to the EST clone, which increased the spacing between motifs A and B' (18) and put all seven RT motifs and the telomerase-specific motif T motifs in a single contiguous ORF (Fig. 2).

RT-PCR amplification of RNA from 293 cells and from testis each gave two products differing by 182 bp (20). The larger and smaller products from testis RNA were sequenced and found to correspond exactly to pGRN121 and the EST cDNA, respectively.

The relative abundance of hTERT mRNA was assessed in six telomerase-negative mortal cell strains and six telomerase-positive immortal cell lines (21) (Fig. 3). The steady-state level of hTERT mRNA was higher in immortal cell lines with active telomerase (6) than in any of the telomerase-negative cell strains tested. Telomerase activity was more strongly correlated with the abundance of hTERT mRNA than with that of telomerase RNA

(hTR) (7). In contrast, the abundance of mRNA for the human p80 homolog TP1 (9) did not correlate with telomerase activity (Fig. 3). Thus, while our proposal that hTERT is the catalytic subunit of human telomerase is based mainly on protein structural features

Table 1. Amino acid sequence identity between telomerase reverse transcriptases. Each value is % identity (% similarity in parentheses) based on RT motifs 1, 2, and A through E in Fig 2C.

	hTERT	SpTrt1p	Est2p
Ea p123	26 (49)	28 (45)	24 (46)
Est2p	25 (46)	27 (48)	
SpTrt1p	30 (47)		

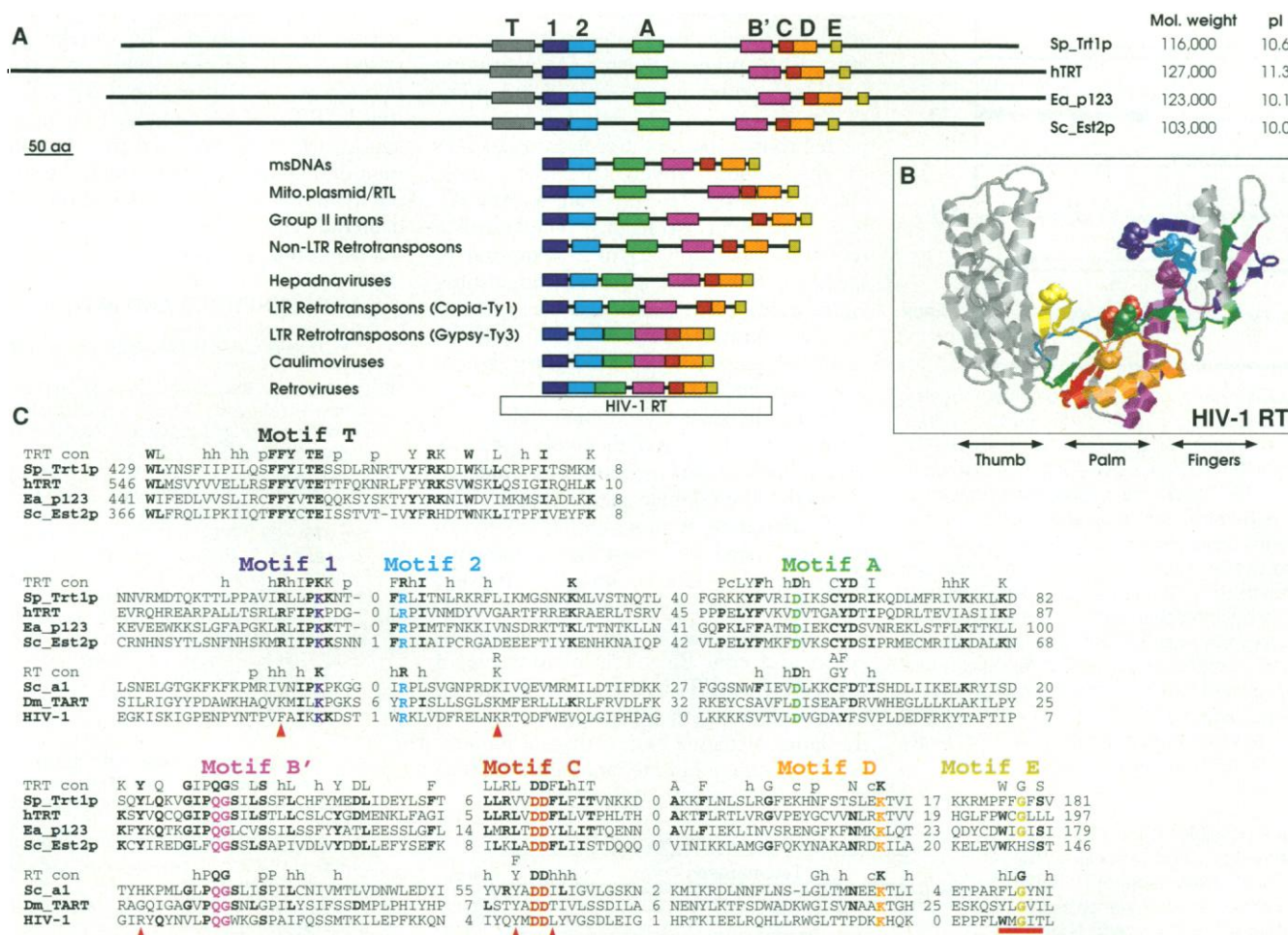


Fig. 2. Structure and RT sequence motifs of telomerase proteins. (A) Locations of telomerase-specific motif T and conserved RT motifs 1, 2, and A through E (24) are indicated by colored boxes. The open rectangle labeled HIV-1 (Human Immunodeficiency Virus) RT delineates the portion of this protein shown in (B). *pI*, isoelectric point. (B) The crystal structure of the p66 subunit of HIV-1 RT (Brookhaven code 1HNV). Color-coding of RT motifs matches that in (A). The view is from the back of the right hand, which allows all motifs to be seen. (C) Multiple sequence alignment of telomerase RTs and members of other RT families (Sc_a1, cytochrome oxidase group II intron 1-encoded protein from *S. cerevisiae* mitochondria; Dm_TART, reverse transcriptase from *Drosophila melanogaster* TART non-LTR retrotransposable element). Boldface residues indicate identity of at least three telomerase sequences in the alignment. Colored residues are highly con-

served in all RTs and shown as space-filled residues in (B). The number of amino acids between adjacent motifs or to the end of the polypeptide is indicated. TRT con and RT con, consensus sequences for telomerase RTs (this work) and non-telomerase RTs (24) (amino acids are designated h, hydrophobic, A, L, I, V, P, F, W, M; p, polar, G, S, T, Y, C, N, Q; c, charged, D, E, H, K, R). Red arrowheads show some of the systematic differences between telomerase proteins and other RTs. Red rectangle below motif E highlights the primer grip region discussed in the text. Abbreviations for the amino acids are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. The nucleotide sequences of the *S. pombe* *trt1+* gene and the human TRT cDNA (pGRN121) have been deposited in GenBank (accession nos. AF015783 and AF015950, respectively).

(similarity of RT motifs, the T motif, molecular mass > 100 kD, $pI > 10$), the correlation of its mRNA expression level with activity also supports this conclusion.

Sequence alignment of the four telomerase genes revealed features similar to other reverse transcriptases, as well as differences that serve as hallmarks of the telomerase subgroup. The new T motif is one telomerase-specific feature not found in the other RTs examined. Another is the distance between motifs A and B', which is

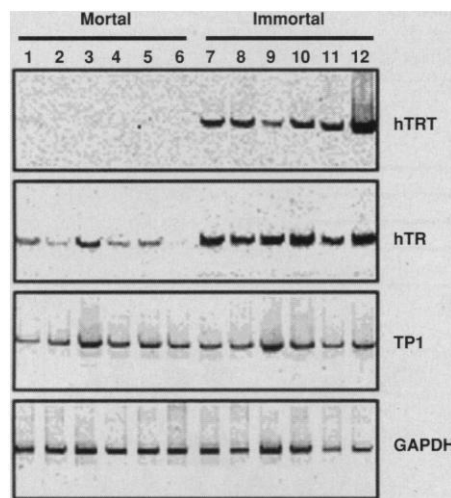
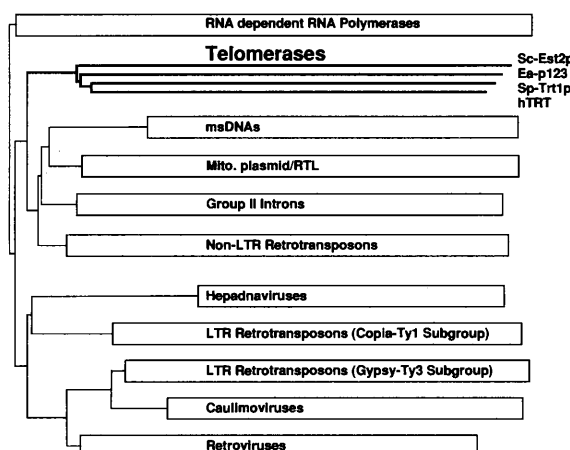


Fig. 3. Expression of hTERT in telomerase-negative mortal cell strains (lanes 1 to 6) and telomerase-positive immortal cell lines (lanes 7 to 12). RT-PCR (21) for hTERT, hTR (human telomerase RNA component), TP1 (telomerase-associated protein related to *Tetrahymena* p80), and GAPDH (to normalize for equal amounts of RNA template) was carried out on RNA from: (1) human fetal lung fibroblasts GFL, (2) human fetal skin fibroblasts GFS, (3) adult prostate stromal fibroblasts 31YO, (4) human fetal knee synovial fibroblasts HSF, (5) neonatal foreskin fibroblasts BJ, (6) human fetal lung fibroblasts IMR90, (7) melanoma LOX IMVI (8) leukemia U251, (9) NCI H23 lung carcinoma, (10) colon adenocarcinoma SW620, (11) breast tumor MCF7, and (12) human 293 cells.

Fig. 4. A possible phylogenetic tree of telomerases and retroelements rooted with RNA-dependent RNA polymerases. After sequence alignment of motifs 1, 2, and A through E (178 positions, Fig. 2C) from four TRTs, 67 RTs, and three RNA polymerases, the tree was constructed using the Neighbor Joining method (30). Elements from the same class that are located on the same branch of the tree are simplified as a box. The length of each box corresponds to the most divergent element within that box.



longer in the TRTs than in other RTs (Fig. 2A). These amino acids can be accommodated as an insertion within the "fingers" region of the structure that resembles a right hand (22, 23) (Fig. 2B). Within the motifs, there are a number of substitutions of amino acids (red arrowheads in Fig. 2C) that are highly conserved among the other RTs. For example, in motif C the two aspartic acid residues (DD) that coordinate active site metal ions (22) occur in the context $hxDD(F/Y)$ in the telomerase RTs compared to $(F/Y)xDDh$ in the other RTs (24). Another systematic change characteristic of the telomerase subgroup occurs in motif E, where $WxGxSx$ appears to be the consensus among the telomerase proteins, whereas $hLGxxh$ is characteristic of other RTs (24). This motif E is called the "primer grip" (23), and mutations in this region affect RNA priming but not DNA priming (25). Because telomerase uses a DNA primer, the chromosome 3' end, it is not unexpected that it should differ from other RTs in this region. Given that the simple change from Mg^{2+} to Mn^{2+} allows HIV RT to copy a small region of a template in a repetitive manner (26), it is tempting to speculate that some of the distinguishing amino acids in the TRTs may cause telomerase to catalyze repetitive copying of the template sequence of its tightly bound RNA subunit.

Using the seven RT domains (Fig. 2C) defined by Xiong and Eickbush (24), we constructed a phylogenetic tree that includes the four telomerase RTs (Fig. 4). The TRTs appear to be more closely related to RTs associated with msDNA (multicopy single-stranded DNA), group II introns, and non-LTR (Long Terminal Repeat) retrotransposons than to the LTR-retrotransposon and viral RTs. The relationship of the telomerase RTs to the non-LTR branch of retroelements is intriguing, given that the latter elements have replaced telomerase for telomere maintenance in *Drosophila*

(27). However, the most striking finding is that the TRTs form a discrete subgroup, about as closely related to the RNA-dependent RNA polymerases of plus-stranded RNA viruses such as poliovirus as to retroviral RTs. In view of the fact that the four telomerase genes are from evolutionarily distant organisms—protozoan, fungi, and mammals—this separate grouping cannot be explained by lack of phylogenetic diversity in the data set. Instead, this deep branching suggests that the telomerase RTs are an ancient group, perhaps originating with the first eukaryote.

The primary sequence of hTERT and eventual reconstitution of active human telomerase may be used to discover telomerase inhibitors, which in turn will permit additional testing of the anti-tumor effects of telomerase inhibition. The correlation between hTERT mRNA levels and human telomerase activity shown here indicates that hTERT also has promise for cancer diagnosis. With an essential protein component of telomerase now in hand, the stage is set for more detailed investigation of fundamental and applied aspects of this ribonucleoprotein enzyme.

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14. PCR primers were based on motif B' (YARACHAARGGHATYCOHYARGG) and motif C (NGTNATDARDARRTARTCRTC), where D = G, A, or T; H = T, A, or C; Y = T or C; R = A or G; and N = G, A, T or C. Approximately 10^6 colonies of an *S. pombe* Hind III-cut genomic library in pWH5 [A. Wright, K. Maundrell, W. D. Heyer, D. Beach, P. Nurse, *Plasmid* **15**, 156 (1986)] and 10^5 colonies of an *S. pombe* cDNA library [J.-P. Javerzat, G. Cranstorn, R. C. Allshire, *Nucleic Acids Res.* **24**, 4676 (1996)] were screened.

15. These cDNA clones terminated in the exon flanked by introns 4 and 5. Poly A⁺ RNA from *S. pombe* was reverse transcribed using primer M2-B14 (CCTTG-GAAAAATCCATTTGAAGCCACATGTG). The resulting cDNA was ligated to oligonucleotide pGGGCCGT-GTTGGCCTAGTCTCTGCTCddA using T4 RNA ligase and amplified by two rounds of PCR: in the first round, we used primers M2-B14 and Adapt-Sfi (GAGGAGGAGAAGAGCAGAGAACTAGGCCAACACCGGCC), and in the second, we used primers M2-B15 (AAAGTGGTATGCCAGAAATCTGAAGG-TAAT) and Adapt-Sfi.
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18. Clone 712562 was obtained from the I.M.A.G.E. (Integrated Molecular Analysis of Genomes and Their Expression) Consortium [G. Lennon, C. Auffray, M. Polymeropoulos, M. B. Soares, *Genomics* **33**, 151 (1996)]. This clone did not encode a contiguous portion of a TRT because motifs B', C, D, and E were contained in a different ORF than the more NH₂-terminal motifs. In addition, the distance between motifs A and B' was substantially shorter than that of the other three TRTs.
19. A lambda cDNA library from the human 293 cell line, which has high levels of telomerase activity, was partitioned into 25 pools containing ~200,000 plaques each. These were screened by PCR with primers LT5 (CGGAAGAGTGTCTGGAGCAA) and LT6 (GGATGAAGCGAGTCTGGA) to the 5' region of the #712562 clone insert. Six subpools of one positive primary pool were further screened by PCR. One positive subpool was then screened by plaque hybridization with a probe from the 5' region of clone #712562. One phage was positively identified and the ~4 kbp insert from this clone was excised and subcloned into the pBluescript II SK⁺ vector (Stratagene) as an Eco RI fragment.
20. Polyadenylated RNAs from human testis and from the 293 cell line were amplified using a nested PCR strategy. The first primer set was TCP1.1 (GTGAAG-GCACTGTCAGCG) and TCP1.15 (CGCGTGGGT-GAGGTGAGGTG); the second primer set was TCP1.14 (CTGTGCTGGGCGTGGAGGATA) and bTCP6 (AGCTTGTCTCCATGTCGCCGTAG).
21. hTRT mRNA was amplified using oligonucleotide primers LT5 and LT6 (79) for 31 cycles (94°C for 45 s, 60°C for 45 s, 72°C for 90 s). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was amplified using primers K136 (CTCAGACACCAT-GGGGAAGGTGA) and K137 (ATGATCTTGAG-GCTGTTGTCATA) for 16 cycles (94°C for 45 s, 55°C for 45 s, 72°C for 90 s). hTR was amplified using primers F3b (TCTAACCCCTAAGTGAAGGGCG-TAG) and R3c (GTTTGCTCTAGAATGAACGGTG-GAAG) for 22 cycles (94°C for 45 s, 55°C for 45 s, 72°C for 90 s). TP1 mRNA was amplified using primers TP1.1 (TCAAGCCAAACCTGAATCTGAG) and TP1.2 (CCCGAGTGAATCTTTCTACGC) for 28 cycles (cycles same as for hTRT). Reaction products were resolved on an 8% polyacrylamide gel, stained with SYBR Green I (Molecular Probes, Eugene, OR).
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28. h⁺/h⁻ *leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M210/ade6-M216 his3-D1/his3-D1 trt1⁺/trt1⁻:his3⁺*.
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Contrasting Genetic Influence of CCR2 and CCR5 Variants on HIV-1 Infection and Disease Progression

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The critical role of chemokine receptors (CCR5 and CXCR4) in human immunodeficiency virus-type 1 (HIV-1) infection and pathogenesis prompted a search for polymorphisms in other chemokine receptor genes that mediate HIV-1 disease progression. A mutation (CCR2-64I) within the first transmembrane region of the CCR2 chemokine and HIV-1 receptor gene is described that occurred at an allele frequency of 10 to 15 percent among Caucasians and African Americans. Genetic association analysis of five acquired immunodeficiency syndrome (AIDS) cohorts (3003 patients) revealed that although CCR2-64I exerts no influence on the incidence of HIV-1 infection, HIV-1-infected individuals carrying the CCR2-64I allele progressed to AIDS 2 to 4 years later than individuals homozygous for the common allele. Because CCR2-64I occurs invariably on a CCR5-+-bearing chromosomal haplotype, the independent effects of CCR5-Δ32 (which also delays AIDS onset) and CCR2-64I were determined. An estimated 38 to 45 percent of AIDS patients whose disease progresses rapidly (less than 3 years until onset of AIDS symptoms after HIV-1 exposure) can be attributed to their CCR2-+/+ or CCR5-+/+ genotype, whereas the survival of 28 to 29 percent of long-term survivors, who avoid AIDS for 16 years or more, can be explained by a mutant genotype for CCR2 or CCR5.

The nexus of chemokine immunobiology and AIDS pathogenesis has revealed untapped avenues for resolving patterns of HIV-1 disease progression, for clarifying epidemiologic heterogeneity, and for design of therapies (1-6). Identification of the CC-chemokines, RANTES, MIP1α and MIP1β, as suppressor factors produced by CD8 cells that counter infection by certain HIV-1 strain infections (7) previewed the critical identification of two chemokine receptor molecules, CXCR4 (formerly named LESTR/fusin) and CCR5 (formerly CKR5), as cell surface coreceptors with CD4 for HIV-1 infection (8-13). Additional chemokine receptors CCR2 and CCR3 also

have been implicated as HIV-1 coreceptors on certain cell types (12-14). HIV-1-infected patients harbor predominantly macrophage-tropic HIV-1 isolates during early stages of infection, but accumulate increasing amounts of T cell-tropic strains just before accelerated T cell depletion and progression to AIDS. The identification of "dual"-tropic HIV-1 strains over the course of infection suggests that such strains may represent an intermediate between macrophage- and T cell-tropic populations (11-13, 15). This tropic transition indicates that viral adaptation from CCR5 to CXCR4 receptor use may be a key step in progression to AIDS (16).