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Expression of mouse telomerase catalytic subunit in embryos and adult tissues

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ABSTRACT **Telomerase is a ribonucleoprotein complex that elongates telomeres, allowing the stable maintenance of chromosomes during multiple cell divisions. Here, we describe the isolation and characterization of the catalytic subunit of mouse telomerase, mTERT (mouse telomerase reverse transcriptase), an essential protein component of the telomerase complex. During embryonic development, mTERT mRNA is abundantly expressed in the whole embryo, especially in regions of intense proliferation. We found that the mTERT mRNA expression in both embryonic and adult tissues is independent of the essential RNA component of telomerase, mTR, and therefore, of the formation of active telomerase complexes. mTERT protein is present exclusively in tissues with telomerase activity, such as testis, spleen, and thymus. mTERT protein is barely detectable in the thymus of mTR$^{-/-}$ mice, suggesting that mTERT protein stability in this tissue may depend on the actual assembly of active telomerase complexes. Finally, we found that mouse and human telomerase catalytic subunit is located in the cell nucleus, and its localization is not regulated during cell cycle progression.**

Telomeres are special structures at the end of chromosomes that protect the integrity of eukaryotic chromosomes (1, 2). Telomeres consist of tandem repeats of a short DNA sequence, which in vertebrates is TTAGGG (3). In the absence of a compensating mechanism, telomeres are progressively shortened because of the inability of conventional DNA polymerases to replicate 3′-terminal sequences (1). Indeed, the chromosomes of most adult human and murine cells undergo telomere shortening at a rate of approximately 120 bp per cell division (2, 4, 5). There are mechanisms that counteract telomere shortening, which operate mainly in cells that do not have a limited lifespan, such as germ-line cells, immortal cell lines, and tumor cells (6–8).

The activity of the enzyme telomerase is the best understood mechanism to maintain telomere length (2, 4, 9–13). Telomerase is a DNA polymerase that in vertebrates elongates the 3′ end of preexisting telomeres by synthesizing TTAGGG sequences, using an internal RNA molecule as template (1, 3). The RNA component of telomerase has been characterized in a number of organisms, including ciliates, yeast, mouse, and human (12, 14–16). Yeast and mouse knock-out strains lacking the telomerase RNA component (termed mTR for the mouse) are completely deficient for telomerase function (2, 12, 13); these telomerase-deficient organisms are viable only for a limited number of generations. In the case of telomerase-deficient mice, telomeres are progressively shortened from one generation to the next; this is accompanied by a progressive increase in chromosomal aberrations and sterility by the sixth generation (2, 17). It is possible, however, to select viable yeast populations deficient in the telomerase RNA component that maintain their telomeres through a recombination-dependent mechanism (13). There are also mammalian tumor cells and mammalian cells immortalized in culture that maintain their telomeres through telomerase-independent mechanisms that are not yet well defined (18).

The protein component of telomerase containing the active polymerization site has recently been isolated from the ciliate *Euplotes aediculatus*, the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, and humans (19–24). These telomerase proteins range in size from 103 kDa in yeasts to 127 kDa in humans. Interestingly, telomerase proteins contain all the conserved amino acid motifs that are important for other reverse transcriptases. In particular, the three aspartic acids that are involved in nucleotide binding and catalysis in conventional reverse transcriptases are also essential for telomerase activity (19, 25, 26). In addition to the telomerase catalytic subunit, several proteins are associated with telomerase activity in ciliates and mammals that could have structural or regulatory roles (23, 27–30).

To characterize the regulation of mammalian telomerase in embryonic and adult tissues, we isolated the mouse telomerase catalytic subunit, and we have generated antibodies specific for the murine and human telomerase catalytic subunits.

MATERIALS AND METHODS

Cloning of Mouse Telomerase Reverse Transcriptase cDNA. The expressed sequence tag database dbEST was searched by using the TBLAST program for sequences homologous to the previously described telomerase reverse transcriptases of *Euplotes aediculatus and Saccharomyces cerevisiae*. Two putative human homologues were found, EST-A [GenBank accession no. AA281296 (also reported by Meyerson *et al.* in ref. 21)] and EST-B (AA311750).

A cDNA library from mouse embryonic stem cells was screened simultaneously with two different PCR fragments derived from EST-A and EST-B, respectively.

EST-A was obtained from Genome Systems (clone 712562A) and PCR-amplified by using the primers EST-A-5′ (5′-GGGGAATTCGCCAAGTTCCTGCACTGGCTGATG-3′) and EST-A-3′ (5′-CCCCCCCTGCAGCTACGCCCGCTCGTAGTTGAGCACGCT-3′) and was used to probe a mouse cDNA library. A second probe was obtained by amplifying a human cDNA library with primers EST-A-5′ (see above) and EST-B-3′ (5′-CCCCCCCTGCAGCTAAGGGAAGTTCACCACTGTCTTCCG-3′). These primers amplify a 1.1-kb fragment that extends from EST-A to EST-B. Both PCR fragments were labeled by random priming extension using labeled [α-^{32}P]dCTP and [α-^{32}P]-dGTP (3000 Ci/mmol; 1 Ci = 37 GBq).

Abbreviations: mTERT and hTERT, mouse and human telomerase reverse transcriptase; mTR and hTR, mouse and human telomerase RNA components; MEFs, mouse embryo fibroblasts; RT-PCR, reverse transcriptase–PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Data deposition: The mTERT sequence reported in this paper has been deposited in the GenBank database (accession no. AF073311).

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The 5′ end of the mouse telomerase reverse transcriptase (mTERT) ORF was obtained by 5′RACE-PCR (GIBCO/BRL, version 2). Total RNA was obtained from mouse embryonic fibroblasts (MEFs) (31). The primers used were PRIMER 5 race1 (5′-AGGTGGAGGCTGTGAGA-3′) for cDNA synthesis, and two nested primers NESTED1 (5′-GGGGTCGACTT-GGGCAACCAAAGTGCG-3′) and NESTED2 (5′-GGTC-GACGCGGTAGATCTTCGGGTC-3′) for the PCR step.

Sequencing was done with a T7 Sequenase version 2.0 kit (Amersham) and an Applied Biosystems 377 DNA sequencer with the Prism dRhodamine Terminator Cycle Sequencing kit (Perkin-Elmer). Sequences were aligned with the MEGAALIGN DNA Star program.

Generation of Antibodies Against mTERT and the Human Analogue hTERT. The antigenic peptide FQKNRLFFYRKS-VWC, also called PEPT-1, was synthesized on an automated multiple peptide synthesizer (AMS 422, Abimed) (32). The peptide was cleaved from the resin (33) and purified in reverse-phase HPLC. Peptide purity and composition were confirmed by reverse-phase HPLC and by amino acid analysis (Beckman 6300).

The peptide was used as immunogen to induce antibodies specific for human and mouse telomerase. Sera were collected and the IgG fraction was purified by protein G-Sepharose chromatography and used for the experiments described. The antibody was called K-370.

Cells and Primary Tissues. Mouse embryo fibroblasts (MEFs) were prepared from 13.5-day wild-type or $mTR^{-/-}$ embryos (2). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

HeLa cells were synchronized by the double thymidine block method (34). MEFs were incubated for 48 hr in low-serum (0.1%) medium, then changed to rich medium (10% serum) plus aphidicolin (5 μg/ml) for 24 hr; cells were released from the block by adding rich medium without the drug. Progression into S phase was monitored by fluorescence-activated cell sorter (FACS) analysis at different times after the block release.

Adult mouse tissues were obtained from 2-month-old strain C57BL/6J or from wild-type and $mTR^{-/-}$ littermates (2).

TRAP Assay and *in Vivo* Reconstitution of Telomerase Activity. Telomerase activity was reconstituted *in vivo* in $mTR^{-/-}$ cells by conventional calcium phosphate transfection with a plasmid containing an empty vector, the hTR gene under a cytomegalovirus (CMV) constitutive promoter, or a plasmid containing the mTR gene and upstream promoter sequences (2). Transfection was carried out as described (2). To measure telomerase activity, S-100 extracts were prepared from cell cultures as described (35). Telomerase activity was measured with a modified version (35) of the TRAP assay (TRAPeze kit; Oncor) (36). An internal control for PCR efficiency was included.

Reverse Transcriptase (RT)-PCR. For RT-PCR analysis of mTERT mRNA, 1 μg of total RNA isolated from cells or tissues (31) was treated with DNase I (Boehringer) and hybridized to 100 ng of 6-nt random hexamers at 95°C for 10 min, followed by reverse transcription at 37°C for 1 h with SuperScript II reverse transcriptase (GIBCO/BRL). As a control, reactions were also carried out in the absence of reverse transcriptase (not shown). PCR amplification was performed using two sets of primers: clone2a (5′-CAGACATTTCCTTTACTC-3′) and clone2b (5′-ACCATATACCTGCCAGGG-3′) or Race I (5′-CTGCAATG-TGACCTGAGG-3′) and 55/1 (5′-GAGCGCAACGAGAGA-AACG-3′). After the PCR cycles (1 min at 94°C, 45 sec at 57°C, and 1 min and 30 sec at 72°C) in the presence of [α-^{32}P]dATP and [α-^{32}P]dGTP, the PCR products were separated in nondenaturing 4% polyacrylamide gels. The number of cycles used was in the linear range and varied from 20 to 25 cycles in different PCRs. To normalize the amounts of input RNA, amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA was used as control.

Whole-Mount *in Situ* Hybridization. A partial mTERT cDNA fragment (nucleotides 1629–3367), cloned in pBluescript SK (−), was digested at the 5′ or 3′ end of the insert and transcribed in the presence of digoxigenin (Riboprobe Kit; Promega) with T7 or T3 RNA polymerase, respectively. Mouse embryos were obtained at days 9–12 from wild-type or $mTR^{-/-}$ pregnant females and fixed in 4% paraformaldehyde at 4°C for 12 hr. Embryos were treated with 6% H_2O_2 for clearing. Embryos were then prehybridized at 70°C for 1 hr (50% formamide, 5× SSC, 0.1 mg/ml yeast RNA, 1% SDS, and heparin), followed by the addition of the probe and incubation at 70°C for 16 hr in the same solution. Embryos were washed and transferred to blocking solution containing 1% Tween 20, 20% fetal bovine serum, and 1× blocking powder. They were then incubated with anti-digoxigenin antibody (anti-digoxigenin Fab fragments coupled to alkaline phosphatase) for 16 hr at 4°C, and the signal was developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; Boehringer Mannheim).

Western Blot Analysis Using K-370 Telomerase-Specific Antibodies. As indicated, 20–100 μg of total protein from S-100 extracts (see below) was loaded per lane and subjected to electrophoresis in SDS/polyacrylamide gels (37). Equal amounts of protein were loaded. To determine protein concentration accurately in the extracts, the Micro BCA (bicinchoninic acid) protein assay kit (Pierce) was used. Gels were transferred to Problott membranes (Applied Biosystems) by using a Mini Trans Blot System (Bio-Rad). Membranes were blocked and incubated with rabbit serum samples for 90 min at room temperature, followed by horseradish peroxidase-labeled protein G and developed with enhanced chemiluminescence (ECL; Amersham). Antibody specificity was determined by immunoblotting using preimmune serum, anti-peptide antiserum, and anti-peptide antiserum preincubated with the immunizing peptide (PEPT-1) or with an unrelated peptide of the same length (PEPT-2)(TFLRTLVRGVPEYGC).

Immunofluorescence. Human HeLa cells, MEFs, and human IMR-90 cells were seeded in chamber slides (Lab-Tek) and cultured overnight in DMEM supplemented with 10% fetal bovine serum. Cells were washed twice in PBS and fixed for 3 min in methanol. After two more washes in PBS, cells were blocked with 10% goat serum (Sigma) and incubated for 1 hr at 37°C with a 1/1000 dilution of the telomerase-specific K-370 antibody in 1% serum. As specificity control, the K-370 antibody was preincubated with the immunizing peptide (see above, not shown). Cells were washed in PBS, and a 1/400 dilution of rhodamine-conjugated anti-rabbit-immunoglobulin (Pierce) in 1% serum was added for 1 hr at 37°C. Nuclei were stained with Hoechst 33258. Images were captured with a Leitz DMRB fluorescence microscope (Leica) and the program Q-FISH (Leica).

Cytoplasmic and Nuclear Extracts. Nuclear and cytoplasmic extracts were prepared from asynchronous and synchronous cultures (1–2 × 10^6 cells) (38). Equal amounts of cytoplasmic and nuclear extracts were loaded per lane for Western blot analysis.

RESULTS

Functional Conservation Between Mouse and Human Telomerases. To isolate mTERT, we used two human expressed sequence tags that presented significant homology with the telomerase reverse transcriptases of ciliates and yeast to screen a mouse cDNA library (*Materials and Methods*). The mTERT gene contains an ORF that encodes a protein of 1,122 amino acids with a predicted molecular mass of 125 kDa.

The full sequence of the human telomerase reverse transcriptase, hTERT, has been reported by several groups (19–24). The nucleotide sequences of mTERT and hTERT are 74% identical, the protein identity is 64%, and the protein similarity, 69%. Mouse and human telomerase RNAs, mTR and hTR, respectively, are also relatively conserved (15). The high degree of identity between murine and human telomerase components suggested that they might share a similar structure. To test whether mouse and human telomerase complexes are functionally conserved, we asked whether the mTERT was able to

reconstitute telomerase activity with the human telomerase RNA subunit *in vivo*. Murine cells that are genetically deficient for the mTR gene, $mTR^{-/-}$, are also deficient for telomerase activity (2). Telomerase activity in these cells can be rescued, however, by expressing an exogenous mTR gene (2) (Fig. 1). To test whether hTR was able to associate with mTERT and form a functionally active telomerase complex, we transfected the hTR gene into $mTR^{-/-}$ cells. hTR was able to reconstitute telomerase activity *in vivo* in mouse cells lacking the mTR gene (Fig. 1), indicating that human and mouse telomerase proteins are functionally interchangeable and suggesting that mouse and human telomerase complexes are structurally conserved.

mTERT mRNA Expression in Embryos. To analyze mTERT expression in mouse embryos, we performed whole-mount *in situ* hybridization using 9- to 12-day embryos and a digoxigenin-labeled mTERT RNA probe (see *Materials and Methods*). Fig. 2 *a* and *b* shows two 11-day wild-type embryos probed with sense or antisense mTERT RNA. The sense probe does not stain the embryos except for a low background signal (Fig. 2*a*). In contrast, the antisense probe shows that mTERT mRNA is abundant in the entire embryo and particularly in limbs, tip of the tail, and the nares (Fig. 2*b*; see also Fig. 2*f*). This expression pattern suggests that mTERT mRNA is expressed at higher levels in regions of intense proliferative activity. At day 12, the nares show a spotted pattern that coincides with the developing hair follicles of the whiskers (see Fig. 2 *c* and *d*, for embryos hybridized with sense or antisense probes, respectively).

To study whether the regulation of mTERT mRNA depends on the presence of other components of the telomerase complex, we analyzed mTERT expression in embryos lacking the essential mTR telomerase component. Embryos deficient in the mouse telomerase RNA gene, $mTR^{-/-}$, were hybridized with mTERT-derived sense and antisense riboprobes. Fig. 2*e* (sense probe) and *f* (antisense probe) show that $mTR^{-/-}$ embryos have a pattern of mTERT mRNA expression similar to that in wild-type embryos (compare Fig. 2 *b* and *f*). These results suggest that mTERT mRNA expression is independent of the presence or absence of mTR, and consequently of the formation of active telomerase complexes.

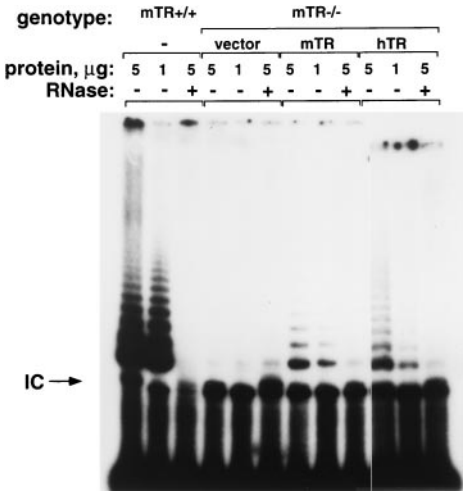

FIG. 1. Rescue of telomerase activity in an $mTR^{-/-}$ cell line with the human telomerase RNA. A plasmid with the hTR gene under a constitutive promoter, or with 5 kb of mouse genomic DNA that contained the mTR gene and upstream sequences (15), or an empty vector (Bluescript) were transfected into a $mTR^{-/-}$ cell line (KO-3 at passage 23). Forty-eight hours after transfection, S-100 extracts were prepared and assayed for telomerase activity. As a control of the TRAP assay, telomerase activity was detected in wild-type ($mTR^{+/+}$) cells. All the extracts were pretreated (+) or not (−) with RNase before the telomerase assay. The protein concentration in the PCR step of the TRAP assay is given in $\mu g/\mu l$. The arrow indicates the position of the internal control (IC) for PCR efficiency.

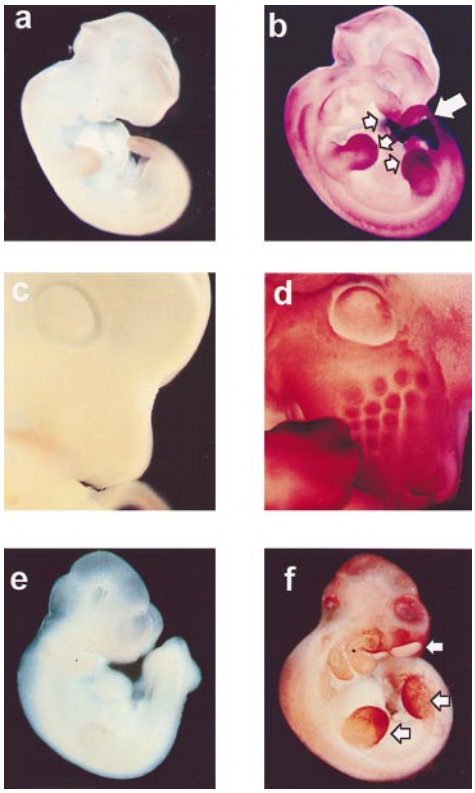

FIG. 2. Expression of mTERT mRNA in mouse embryos. (*a* and *b*) Eleven-day wild-type embryos hybridized with sense (*a*) or antisense (*b*) mTERT-derived riboprobes. *b* shows generalized expression of mTERT. Regions of particularly high levels of mTERT mRNA are indicated with arrows. (*c* and *d*) Detail of the head of a 12-day wild-type embryo hybridized with sense (*c*) or antisense (*d*) mTERT-derived riboprobes. d shows high levels of mTERT mRNA in the developing hair follicles of the nares. (*e* and *f*) Ten-and-one-half-day $mTR^{-/-}$ embryos hybridized with sense (*e*) or antisense (*f*) mTERT-derived riboprobes. The pattern and intensity of the mTERT mRNA signal are similar in *b* and *f*.

mTERT mRNA Expression in Adult Tissues. We studied mTERT mRNA expression in adult murine tissues by RT-PCR using two sets of primers that amplify fragments of 363 and 988 bp (*Materials and Methods*). In a first set of experiments, we used cDNA derived from four adult tissues: testis and liver, which have telomerase activity, and brain and kidney, which are telomerase-negative or contain low levels of telomerase activity, respectively (5, 39). Using these two sets of mTERT-specific primers, we found that mTERT mRNA is detectable in all tissues, regardless of the presence or absence of telomerase activity, although its abundance appears more prominent in testis and liver, in agreement with the fact that these tissues also show high levels of telomerase activity (Fig. 3). As a control, GAPDH mRNA was detected by RT-PCR, showing equal amounts of GAPDH product in the four tissues tested (Fig. 3*A*).

The fact that mTERT mRNA is detectable in adult tissues lacking detectable telomerase activity suggests that the formation of active telomerase complexes could be subject to further regulation, such as the presence of the telomerase RNA (mTR) and/or posttranscriptional regulation of mTERT. To analyze whether mTERT mRNA expression in adult tissues depends on the presence of mTR, we studied mTERT mRNA expression in adult tissues derived from $mTR^{-/-}$ mice. mTERT mRNA was detected by RT-PCR in three different adult tissues that normally show telomerase activity, spleen, thymus, and liver, independently of the presence ($mTR^{+/+}$ or wt) or absence ($mTR^{-/-}$ or KO) of the mTR component (Fig. 3*B*). This indicates that mTERT mRNA expression is independent of the presence of the mTR component. Testis from wild-type mice had slightly higher

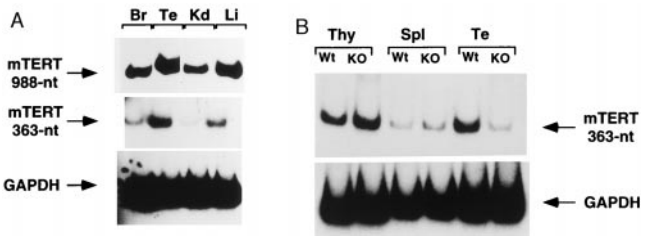

FIG. 3. Expression of mTERT mRNA in adult tissues. Total RNA isolated from the indicated wild-type or $mTR^{-/-}$ mouse tissues was reverse transcribed and then subjected to PCR amplification with two different sets of primers: 998-nt mTERT PCR product was obtained with Race1 and 55/1 primers and a 363-nt product was obtained when clone2a and clone2b primers were used. (*A*) RT-PCR from four tissues derived from 2-month-old wild-type mice. (*B*) RT-PCR from three tissues derived from wild-type (wt) or $mTR^{-/-}$ (KO) littermates. Arrows indicate the RT-PCR fragments obtained with primers against mTERT or GAPDH genes. Br, brain; Te, testis; Kd, kidney; Li, liver; Spl, spleen; Thy, thymus.

amounts of mTERT mRNA than did the corresponding $mTR^{-/-}$ tissue, but the significance of this is not clear, since the mTERT protein levels appear to be similar in the two tissues (see below).

Expression of mTERT Protein in Adult Tissues. We generated antibodies against hTERT and mTERT. Antiserum K-370 was obtained against a synthetic peptide (peptide K-370 or PEPT-1) encoded by *EST1* from human telomerase reverse transcriptase and conserved in mTERT protein.

K-370 antibodies recognize a band in human and mouse immortal cell lines, 293 and FM3A, respectively (Fig. 4), in agreement with the fact that these cells have high telomerase activity levels (40, 41). The protein recognized in human extracts has a lower mobility than that present in mouse extracts, as expected from their predicted molecular masses, 127 kDa for hTERT and 125 kDa for mTERT. In both cases, the signal disappeared after preincubation of the antibody with the antigenic peptide (PEPT-1), but not when a nonspecific peptide (PEPT-2) was used (Fig. 4). Human primary cells IMR-90 do not present detectable amounts of the telomerase reverse transcriptase subunit (Fig. 4; see also Fig. 7*A*), in agreement with the fact that they lack telomerase activity (21). MEFs, with low telomerase activity levels (2), did not show detectable mTERT protein in this assay (Fig. 4); however, we detected mTERT protein in these cells by using a different assay (Fig. 7*A*).

To study mTERT protein expression, S-100 extracts from a number of tissues were prepared and analyzed by Western blotting using K-370 antibody. The mTERT-specific band is detected in telomerase-positive tissues such as thymus, testis, spleen, and liver (Fig. 5*A*) (5, 37) but is undetectable in ovary, muscle, brain, intestine, and heart, in agreement with the fact that all, except ovary, have been described as telomerase-negative or as low telomerase activity (Fig. 5*A*) (5, 40).

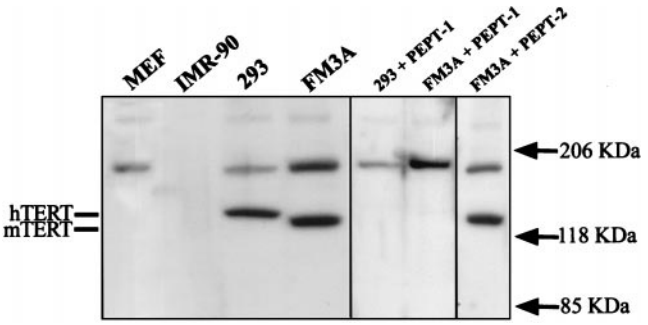

FIG. 4. Characterization of K-370 antibodies against mouse and human telomerase catalytic subunits. Western blot analysis of total cell lysates from the indicated mouse (MEF, FM3A) or human (IMR-90, 293) cells using K-370 antibodies in the absence of competing peptides, or in the presence of the antigenic peptide PEPT-1 or the unrelated peptide PEPT-2. FM3A is a mouse immortal cell line; IMR-90 cells are nonimmortalized human diploid fibroblasts; and 293 cells are adenovirus-transformed human fibroblasts. MEFs had undetectable mTERT protein in this assay and IMR-90 cells are negative for telomerase activity, whereas FM3A, MEFs, and 293 have detectable telomerase activity.

When mTERT protein was analyzed in thymus, spleen, and testis derived from wild-type (wt) or $mTR^{-/-}$ (KO) littermate mice, we observed similar amounts of the mTERT-specific band in testis and spleen in the two genotypes (Fig. 5*B*; note that lane FM3A has 1/4 the protein loaded in Fig. 5*A*). This indicates that both mTERT mRNA (see above) and mTERT protein are expressed independently of the presence of active telomerase complexes in these tissues. In contrast, the $mTR^{-/-}$ thymus lacked detectable mTERT protein, which was, however, present in wild-type thymus (Fig. 5*B*). This observation suggests that the presence of the mouse telomerase RNA component in the thymus may be necessary for the accumulation of mTERT protein and that this kind of regulation may be tissue specific. The fact that the $mTR^{-/-}$ thymus and the wild-type thymus have similar mTERT mRNA levels indicates that the mTERT protein expression is regulated posttranscriptionally in this tissue.

Subcellular Localization and Cell Cycle Regulation of TERT Protein. To determine the subcellular localization of telomerase catalytic subunit in mammalian cells, such as human HeLa and IMR-90 cells, as well as in primary mouse cells (MEFs), we used K-370 telomerase antibody in Western blot analysis and in immunofluorescence.

Fig. 6 shows a double staining of two different asynchronous cell cultures by using Hoechst 33258 to stain the nucleus and K-370 antibody against the telomerase catalytic subunit. IMR-90 cells, previously shown to lack detectable levels of hTERT protein (Fig. 4), were not stained with K-370 antibody (Fig. 6 *a* and *b*, for Hoechst and K-370 staining, respectively). HeLa cells, which have high levels of telomerase activity (41), showed, in contrast, a clear staining of the nucleus (Fig. 6, *c*/*e* and *d*/*f*, for Hoechst and K-370 staining, respectively), and the fluorescent signal was blocked by preincubation of the antibody with the antigenic peptide (PEPT-1) (not shown). The nuclear staining of hTERT showed a punctuated pattern, supporting the previously described nuclear staining in HeLa cells by using an anti-peptide antibody to a different region of the hTERT (23). We are currently developing

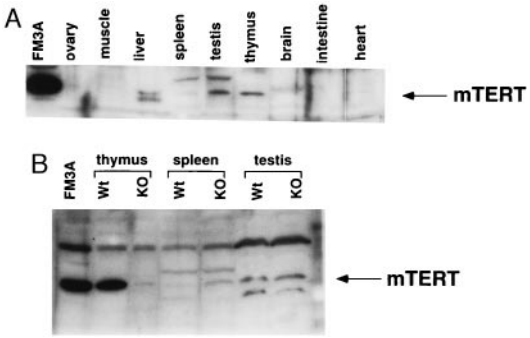

FIG. 5. Expression of mTERT protein in different murine tissues. (*A*) Western blot analysis of S-100 extracts from the indicated tissues, using K-370 antibodies. Tissues were obtained from wild-type mice. One hundred micrograms of total protein was loaded per lane. FM3A is a mouse cell line expressing high levels of mTERT (see Fig. 4). The distortion in the lane corresponding to liver is probably because of the presence of an abundant protein in the relevant region of the gel. Band specificity was confirmed by preincubation of the K-370 antibodies with the antigenic peptide (not shown). (*B*) Western blot analysis of S-100 extracts of thymus, spleen, and testis obtained from wild-type and $mTR^{-/-}$ littermates, and using K-370 antibodies. One hundred micrograms of total protein was loaded per lane, except in the case of the FM3A extract, of which only 20 μg was loaded. The specificity of the mTERT band was determined by preincubation of the K-370 antibody with the antigenic peptide (not shown).

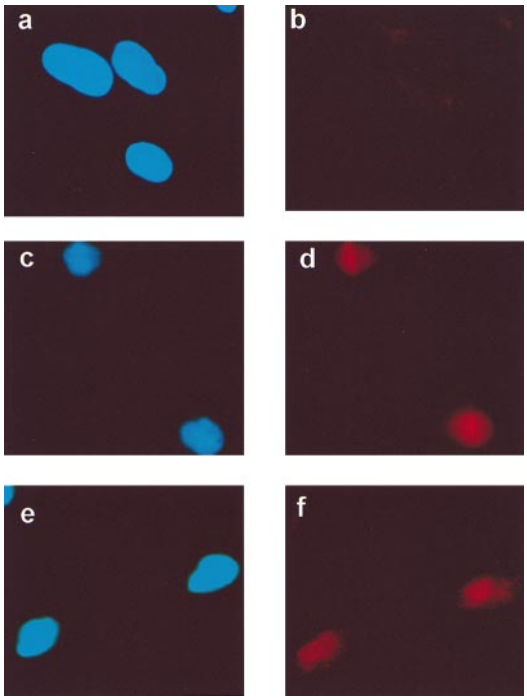

FIG. 6. Subcellular location of human telomerase catalytic subunit. IMR-90 (*a* and *b*) and HeLa (*c*, *d*, *e*, and *f*) cells were simultaneously stained with Hoechst 33258 to visualize nuclei (*a*, *c,* and *e*) and with anti-hTERT antibodies K-370 (*b*, *d*, and *f*). IMR90 are nonimmortal human diploid fibroblasts that have no detectable telomerase activity. HeLa are human papillomavirus-transformed human epithelial cells that have telomerase activity. Note the punctuated hTERT expression pattern in the nucleus. (Objective was ×100.)

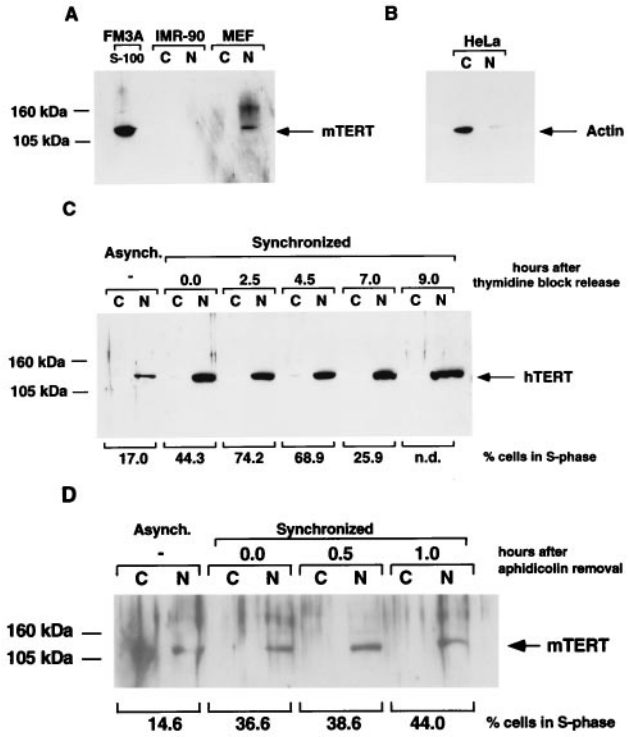

FIG. 7. Cell cycle regulation of TERT protein. (*A*) Fifty micrograms of either nuclear (N) or cytoplasmic (C) extracts from asynchronous populations of IMR-90 and MEFs were analyzed by Western blotting using K-370 antibodies. mTERT was detected exclusively in the nucleus of the MEFs. (*B*) Fifty micrograms of either nuclear (N) or cytoplasmic (C) extracts from asynchronous HeLa cells were analyzed by Western blotting using anti-actin antibodies. The cytoplasmic fraction showed the actin band. (*C*) Western blot analysis, using K-370 antibodies, of nuclear (N) and cytoplasmic (C) extracts from asynchronous or synchronized HeLa cells at the indicated times after the thymidine block release. Fifty micrograms of total protein was loaded per lane. The percentage of cells in S phase at the different points analyzed is also indicated. (*D*) Western blot analysis, using K-370 antibodies, of nuclear (N) and cytoplasmic (C) extracts from asynchronous or synchronized MEFs at the indicated times after the aphidicolin block release. One hundred micrograms of total protein was loaded per lane. The percentage of cells in S phase at the indicated different points analyzed is also shown.

more potent antibodies to asses the nature of the speckles, which may correspond to the telomeres or to replication complexes (42). Mouse primary cells, MEFs, did not show a detectable specific signal in immunofluorescence (not shown), probably because mTERT protein levels in these cells are undetectable or very low (Fig. 4, also Fig. 7*A*). To confirm the nuclear localization of TERT, we prepared nuclear and cytoplasmic extracts from asynchronous cultures of both human (IMR-90 and HeLa) and mouse cells (MEFs) and analyzed the presence of the TERT protein by Western blotting. Equal protein amounts of cytoplasmic and nuclear extracts were analyzed. IMR-90 cells showed no detectable hTERT band, in agreement with the fact that these cells are negative for telomerase activity (Fig. 7*A*, see also Fig. 4). Both human immortal HeLa cells and primary mouse MEFs show expression of TERT protein in the nuclear fraction but not in the cytoplasmic fraction, confirming that TERT protein is located in the cell nucleus (see Fig. 7 *A* and *C*). As a control for the extract preparation, actin was found only in the cytoplasmic fraction of asynchronous HeLa extracts (see Fig. 7*B*).

To analyze whether telomerase activity is regulated by the subcellular localization of TERT protein during cell cycle progression, we studied TERT expression in synchronized HeLa cells and MEFs (Fig. 7 *C* and *D*). The percentage of cells in each cell cycle stage was monitored by FACS analysis. In both cell types, TERT is located in the cell nucleus throughout all the stages of the cell cycle, indicating that TERT availability is not regulated by nuclear transport (Fig. 7 *C* and *D*). The amounts of hTERT protein showed a moderate 3-fold increase as more cells entered the S phase (compare asynchronous cells to 0.0-hr synchronized cells in Fig. 7*C*).

DISCUSSION

The characterization of mouse telomerase activity and of the mouse telomerase RNA component, mTR, and the subsequent construction of the mTR$^{-/-}$ mouse, has established the mouse as a model system to study mammalian telomerase and telomeres (2, 5, 15, 17, 40). To further characterize mouse telomerase regulation, we isolated the mouse telomerase catalytic subunit, mTERT, and generated antibodies to it. The mTERT sequence contains all the amino acid motifs conserved among human, yeast, and ciliate telomerases, including the motifs that are also conserved in the active site of the group of the reverse transcriptases (19–24). The fact that we have reconstituted telomerase activity in mTR$^{-/-}$ cells by introducing the human telomerase RNA, hTR, indicates that mouse and human telomerase complexes are functionally conserved, as the hTR molecule can form active telomerase complexes *in vivo* with both mouse and human TERTs. This is in contrast with previous *in vitro* experiments that showed that mTR is not able to reconstitute telomerase activity with the hTERT subunit (43). These differences could be because of the lack of proper folding of mTR *in vitro*. In this regard, ciliate telomerase RNAs are also interchangeable *in vivo*, *Glaucoma* telomerase RNA being able to reconstitute telomerase activity when expressed in *Tetrahymena* cells (44).

Telomerase activity is not required for the viability of the first generations of mice lacking the essential RNA component of telomerase (mTR) (2, 17). However, as telomeres become critically short in later mTR$^{-/-}$ mouse generations, highly proliferative organs such as the immune system, hematopoietic system,

and the germ-line tissues, which normally express telomerase activity, show defects in their proliferative capacity. Telomerase activity is also expressed during human embryonic development (45), suggesting that telomere maintenance by telomerase could have a role in normal embryonic development. We have taken advantage of the isolation of mTERT cDNAs to study mTERT mRNA expression during embryonic development. mTERT mRNA was expressed in the whole embryo, but significantly enriched in regions with high proliferative activity, suggesting an association between proliferation and telomerase activity during embryonic life. The nares of 12-day embryos showed a spotted mTERT mRNA expression pattern that coincides with the developing hair follicles of the whiskers, in agreement with the fact that telomerase activity is present in adult human hair follicles and skin (46, 47).

To investigate the regulation of telomerase catalytic subunit, we analyzed mTERT mRNA expression in embryonic and adult mouse tissues lacking another essential telomerase component, the mouse telomerase RNA (mTR) (2). $mTR^{-/-}$ embryos and tissues show the same mTERT mRNA expression pattern as their wild-type counterparts. Together, these observations indicate that mTERT mRNA expression during embryonic development and in adult tissues is independent of the expression of the mTR component, and therefore, of the presence or absence of active telomerase complexes.

Similarly, although telomerase activity is detectable in only some adult murine tissues (5, 39), using RT-PCR we detected mTERT mRNA in telomerase-negative tissues such as brain or kidney. In contrast to the mTERT mRNA, mTERT protein was found only in tissues with significant telomerase activity, including thymus, spleen, and testis, but not in brain or kidney, which lack or have low levels of telomerase activity. The fact that we observe mTERT mRNA in tissues in which we do not detect mTERT protein could be because of the greater sensitivity of the method used to detect the mRNA; alternatively, mTERT protein levels may be also regulated by post-transcriptional mechanisms.

mTERT protein is also present in testis and spleen of $mTR^{-/-}$ mice, indicating that both mTERT mRNA and mTERT protein are normally expressed in these tissues in the absence of active telomerase complexes. In the thymus, however, active telomerase complex formation may be a condition for mTERT protein stability, since mTERT protein is absent from the thymus of $mTR^{-/-}$ mice that have normal mTERT mRNA levels.

Finally, we have determined that TERT protein is located in the cell nucleus in both primary and immortal cells and in both mice and humans. Two different cell types, immortal human HeLa cells, which are known to contain high levels of telomerase activity (41), as well as mouse primary cells (MEFs), which show low levels of telomerase activity (2), show TERT in the nucleus. In HeLa cells, hTERT was found in the nucleus and was absent from the cytoplasm in both immunofluorescence and Western blot analysis. Moreover, hTERT seems to be grouped in nuclear speckles reminiscent of those formed by many proteins involved in DNA replication and transcription (42); this hTERT punctuated expression pattern could alternatively correspond to the telomeres. The fact that the majority of HeLa cells and MEFs in an asynchronous population present TERT in their nuclei suggests that nuclear transport is not an important mechanism of telomerase regulation in these cells. In agreement with this idea, we show that TERT is a nuclear protein in both cell types throughout the cell cycle.

The isolation and characterization of the catalytic submit of the mouse telomerase complex and the construction of new mouse models that lack or overexpress this gene will help to further understand the role of telomerase and telomeres in mammals.

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