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Identification of a nonprocessive telomerase activity from mouse cells

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ABSTRACT **Telomerase activity was identified in extracts from several different mouse cell lines. Addition of telomeric TTAGGG repeats was specific to telomeric oligonucleotide primers and sensitive to pretreatment with RNase A. In contrast to the hundreds of repeats synthesized by the human and *Tetrahymena* telomerase enzymes *in vitro*, mouse telomerase synthesized only one or two TTAGGG repeats onto telomeric primers. The products observed after elongation of primers with circularly permuted $(TTAGGG)_3$ sequences and after chain termination with ddATP or ddTTP indicated that mouse telomerase pauses after the addition of the first dG residue in the sequence TTAGGG. The short length of the products synthesized by mouse telomerase was not due to a diffusible inhibitor in the mouse extract, because the human telomerase continued to synthesize long products when mixed with mouse fractions. Primer challenge experiments showed that the human enzyme synthesized long TTAGGG repeats processively *in vitro*, whereas the mouse telomerase appeared to be much less processive. The identification of short telomerase reaction products in mouse extracts suggests that extracts from other organisms may also generate only short products. This knowledge may aid in the identification of telomerase activity in organisms where activity has not yet been detected.**

Telomerase is a highly specialized DNA polymerase which synthesizes telomeric repeat sequences *de novo* both *in vitro* and *in vivo* (reviewed in ref. 1). Net telomere elongation by telomerase may balance the loss of sequences from chromosome ends at each round of DNA replication (2, 3). Telomerase is a ribonucleoprotein in which the RNA component provides the template for the synthesis of telomeric repeats (4, 5). Telomerase activity has been identified in the ciliates *Tetrahymena, Euplotes*, and *Oxytricha* and in transformed human cells (for review see refs. 1 and 6). *In vitro, Tetrahymena* telomerase is highly processive, synthesizing hundreds of telomeric $(TTGGGG)_n$ repeats (7). Telomere lengthening in *Tetrahymena in vivo* appears to be much less processive than primer elongation by telomerase *in vitro* (8), suggesting that telomere length could be regulated in part by telomerase processivity. The large number of yeast genes which affect telomere length (9–13) suggest that length regulation may be a complex process involving telomerase, telomere-binding proteins, and other components.

Telomere length regulation is implicated in both cellular senescence and the immortalization of human cells. In primary somatic cells, telomeric $(TTAGGG)_n$ repeats are lost with age both *in vivo* and *in vitro*. This shortening has been proposed to play a role in signaling the cell cycle exit characteristic of senescent cells (14, 15), although a causal role has not been demonstrated. In contrast to primary human cells, telomere length in immortalized cell lines is stably maintained (16, 17). Recent data (18) have shown that telomerase activity was not detectable in primary human cells when telomeres were shortening. However, immortalized clones arising from simian virus 40 T-antigen-transformed primary cells had both stable telomere length and detectable telomerase activity. This suggests that activation of telomerase may be essential for the growth of immortalized human cells.

To further explore the role of telomerase in cellular senescence and immortalization, we have extended our studies of telomere length and telomerase activity to mice. Mouse telomeres consist of the same $(TTAGGG)_n$ repeated sequence found in humans, although the repeat tracts appear to be much longer in *Mus musculus* than in human cells (19–21). As an initial step in examining what role telomerase might play in the generation and maintenance of the long telomere tracts, we assayed for telomerase in mouse cell extracts and characterized its activity.

MATERIALS AND METHODS

Cell Lines. FM3A cells were a gift from Carol Prives (Columbia University, New York). NS-1 cells (P3/NS1/1-Ag4-1; ATCC no. TIB 18) and the Jurkat cell line (ATCC no. TIB 152) were obtained from the American Type Culture Collection. The YAC-1 (ATCC no. TIB 160) and Sp2/0-Ag14 (ATCC no. CRL 1581) cell lines were gifts from Silvia Bacchetti (McMaster University, Hamilton, ON, Canada).

Cell Extracts. S-100 extracts were prepared according to the human S-100 protocol (18) and then were fractionated on DEAE-agarose columns. Generally, a 1-ml DEAE-agarose column was equilibrated with 0.1 M NaCl in "hypobuffer" [24 mM Hepes, pH 8.0/7 mM KCl/2.4 mM $MgCl_2$/1 mM dithiothreitol/0.1 mM phenylmethanesulfonyl fluoride/1 μM leupeptin/10 μM pepstatin A containing RNase inhibitor (RNasin, 10 units/ml; Promega)], and a 1-ml aliquot of S-100 extract was loaded. The column was washed with 3 volumes of 0.1 M NaCl in hypobuffer, and bound material was eluted in steps with 2 volumes of 0.2 M NaCl in hypobuffer followed by 2 volumes of 0.3 M NaCl in hypobuffer.

Telomerase Assay. An aliquot (20 μl) from each S-100 or DEAE fraction was mixed with 20 μl of 2× reaction buffer (100 mM Tris acetate, pH 8.5/100 mM potassium acetate/4 mM dTTP/4 mM dATP/2 mM $MgCl_2$/2 mM spermidine/2 mM EGTA/10 mM 2-mercaptoethanol), 20 μCi of [α-^{32}P]dGTP (800 Ci/mmol; NEN; 1 Ci = 37 GBq), and 1–2 μM $(TTAGGG)_3$ primer and incubated at 30°C for 60 min. The reactions were stopped with 10 mM EDTA in 5 mM Tris (pH 7.5), and the samples were treated first with RNase A (100 μg/ml) for 15 min at 37°C and then with proteinase K (100 μg/ml) in 0.18% SDS/10 mM Tris (pH 7.5) for 15 min at 37°C and were extracted with phenol. The DNA products were precipitated with ethanol and 5 μg of tRNA and

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separated in an 8% acrylamide/7 M urea denaturing gel as described (18).

Primer Challenge. S-100 extract was prepared from the cell line 293 as described (18), and 110 μl of extract was preincubated with an equal volume of 2× reaction buffer (100 mM Tris acetate, pH 8.5/100 mM potassium acetate/4 mM dTTP/4 mM dATP/2 mM $MgCl_2$/2 mM spermidine/10 mM 2-mercaptoethanol) and 1 μM $(TTAGGG)_3$ primer for 3 min at room temperature to allow binding of primer to telomerase. At time zero 97 μl of [α-^{32}P]dGTP (970 μCi; 800 Ci/mmol; NEN) was added to the preincubation mixture to give a final concentration of 3.8 μM. After incubation at 30°C for 3 min, 110 μl of the reaction mixture was diluted 30-fold into 3.19 ml of 1× reaction buffer (prewarmed to 30°C) containing a final concentration of 17 μM dGTP and either 2 μM $(TTAGGG)_3$ primer or no primer. The remainder of the reaction mixture was not diluted. All reactions proceeded at 30°C, and 1-ml aliquots were removed from the dilution mixtures at 9, 18, and 36 min after reaction initiation (i.e., 6, 15, and 33 min after dilution). The remaining undiluted mixture (34 μl) was allowed to react for a total of 36 min. Reactions were terminated and the products were precipitated and electrophoresed as described (18).

FM3A cell S-100 extracts were purified over a DEAE column as described and a 200-μl aliquot of the active fraction was incubated with an equal volume of 2× reaction buffer (as above) and 1 μM $(GTTGGG)_3$ primer. The reaction was preincubated for 2 min at room temperature, and at time zero 20 μl of [α-^{32}P]dGTP (200 μCi; 800 Ci/mmol; NEN) was added to give a final concentration of 0.62 μM. The reaction mixture was incubated at 30°C for 3 min, and then 160 μl aliquots were diluted 10-fold into 1.44 ml of 1× reaction buffer (prewarmed to 30°C) containing 10 μM dGTP and either 3.3 μM $(GGGGTT)_3$ primer or no primer. Of the remaining undiluted reaction mixture, 40 μl was stopped immediately and 40 μl was terminated at 120 min from time zero. All reactions proceeded at 30°C and 400-μl aliquots were removed from the dilution mixtures and terminated at 15, 30, 60, and 120 min from time zero. The reaction products were extracted with phenol, precipitated, and electrophoresed in an 8% polyacrylamide sequencing gel (18).

Micrococcal Nuclease Digestion. A sample (50 μl) of 293 S-100 extract was incubated for 15 min at 30°C with 100 units of micrococcal nuclease and 1 mM $CaCl_2$. The reaction was stopped by addition of EGTA to 2.5 mM, the mixture was chilled, and $MgCl_2$ was added to 2.5 mM. The reaction mixture was split into three aliquots of 17 μl, and one aliquot was assayed without further treatment. To the remaining two aliquots, 15 μl of FM3A DEAE fraction was added and assayed with or without RNase pretreatment. As a control, 20 μl of 293 S-100 extract was preincubated for 5 min with 1 mM $CaCl_2$ and 2.5 mM EGTA and then treated for 15 min at 30°C with 40 units of micrococcal nuclease. The reaction mixture was chilled and brought up to 2.5 mM $MgCl_2$, and 17 μl was assayed for telomerase activity.

RESULTS AND DISCUSSION

S-100 extracts from the immortalized mouse cell line FM3A were prepared with conditions optimized for human telomerase (22). The crude S-100 extract was loaded onto a DEAE-agarose column and eluted with several steps of increasing NaCl concentration, and all samples were assayed for telomerase activity (data not shown). Although a ladder of TTAGGG repeats typical of human or *Tetrahymena* telomerase products was not observed, one predominant product band was observed above the position of the input primer oligonucleotide. The synthesis of this band was both dependent on input $(TTAGGG)_3$ and sensitive to pretreatment of the extract with RNase A (Fig. 1). Similar nuclease

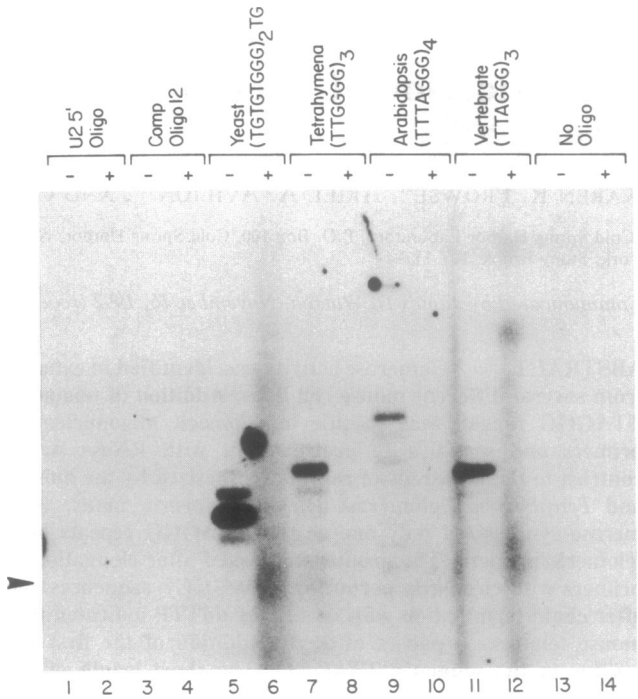

FIG. 1. Characterization of mouse telomerase. The 0.2 M NaCl DEAE fraction from FM3A cells was assayed using different input primer oligonucleotides. Reaction products are shown in pairs without (−) or with (+) RNase A pretreatment, respectively. Lanes 1 and 2, U2 5′(GGCCGAGAAGCGAT); lanes 3 and 4, Comp 12 (AAATCTAGTGCTG); lanes 5 and 6, yeast (TGTGTGGGTGTGTGGGTG); lanes 7 and 8, *Tetrahymena* $[(TTGGGG)_3]$; lanes 9 and 10, *Arabidopsis* $[(TTTAGGG)_3]$; lanes 11 and 12, vertebrate $[(TTAGGG)_3]$; lanes 13 and 14, no input oligonucleotide. Position of a ^{32}P-labeled $(TTAGGG)_3$ is indicated by an arrowhead.

sensitivity has been demonstrated with *Tetrahymena* and other telomerase enzymes (3, 22–24). The product band, however, was insensitive to RNase A. The mouse activity was eluted in 0.2 M NaCl, similar to the elution of human telomerase from a DEAE column (25), and this fraction was used in all subsequent experiments. Optimal $(TTAGGG)_3$ primer labeling was obtained with 1–2 μM primer and 2 mM each dATP and dTTP, and the products continued to accumulate up to 120 min (data not shown). Both the product accumulation and the requirement of the mouse telomerase for high concentrations of primer and nucleotides are similar to features observed for human telomerase (22).

We tested the ability of mouse telomerase to extend both telomeric and nontelomeric oligonucleotides. Incorporation of [α-^{32}P]dGTP onto the telomeric primers $(TTAGGG)_3$, $(TTTAGGG)_4$, $(TTGGGG)_3$, and $(TGTGTGGG)_2TG$ was sensitive to pretreatment with RNase A. The two nontelomeric primers U2 5′ and Comp 12 were not elongated in the extract, and no products were generated in the absence of oligonucleotide (Fig. 1). This shows that the addition of nucleotides is dependent on the input of telomeric primers, and indicates that these primers are recognized specifically by telomerase.

All telomerase enzymes characterized to date can specifically recognize the 3′ end sequence of telomeric primer oligonucleotides and add the correct next base in the telomeric repeat. Because telomerase always pauses during nucleotide addition at the same position within a repeat, different product patterns are generated by using primers with different 3′ ends (3, 22–24). To determine whether the short products generated in the mouse extracts were synthesized by telomerase, three different 18-base primer oligonucleotides containing permutations of the sequence

$(TTAGGG)_3$ were used. The oligonucleotides $(TTAGGG)_3$, $(GGGTTA)_3$, and $(GTTAGG)_3$ primed the addition of specific, yet different-length products (Fig. 2). The product primed by $(TTAGGG)_3$ was four nucleotides longer than the input primer, whereas the product primed by $(GTTAGG)_3$ was five nucleotides longer and that primed by $(GGGTTA)_3$ was only one nucleotide longer. The product length generated with each primer suggested that the correct sequence was added and that primer elongation stopped after the addition of the first dG residue in the sequence TTAGGG.

The incorporation of dideoxynucleotides was used to confirm the nucleotide sequence added onto telomeric primers. In the absence of dideoxynucleotides, the primer $(GTTAGG)_3$ was elongated by five nucleotides, expected to represent the addition of GTTAG. Consistent with this interpretation, the product was one nucleotide shorter when ddATP was added to the reaction mixture. When ddTTP was added, a three-nucleotide decrease in size was seen (Fig. 2). These results indicate that the short products generated by the addition activity in the mouse cell extracts are telomerase elongation products. They further show that mouse telomerase pauses or stops preferentially after the addition of the first dG residue in the sequence TTAGGG. This is the same position in the repeat as the strong pause seen with the human enzyme (22) and analogous to the pausing after the addition by the *Tetrahymena* enzyme of the first dG in the sequence TTGGGG (7).

Telomerase isolated from the ciliates *Tetrahymena*, *Euplotes*, and *Oxytricha* and from human cells can elongate telomeric-sequence oligonucleotides from heterologous species (3, 22–24). Similarly, the mouse telomerase elongated *Tetrahymena* telomere primers, although these products showed the addition of two or three repeats rather than a single repeat. The oligonucleotide $(TTGGGG)_3$ showed product bands of 4 or 10 added nucleotides, $(GGGGTT)_3$ had products of 2, 8, or 14 nucleotides, and $(GTTGGG)_3$ had 4, 10, or 16 nucleotides added (Fig. 2). The ability of the mouse enzyme to synthesize longer products with the $(TTGGGG)_3$ primers may be due to differences in the secondary and tertiary structures of these oligonucleotides (26–29) or to mismatching of the template and primer sequences.

To determine whether the short products synthesized in the mouse extracts were specific to the cell lines used or to species differences between human and mouse, we surveyed a number of different established mouse and human cell lines. In addition to FM3A, S-100 extracts were made from the mouse cell lines NS-1, YAC-1, and Sp2/0-Ag14 (see *Materials and Methods*) and fractionated on DEAE-agarose columns. The $(TTAGGG)_3$-primed elongation products from all of these mouse extracts were short, like those of the FM3A extract described above (data not shown). Human telomerase activity has been assayed in our laboratory and others from at least five different cell lines: HeLa (22), 293, HA1, and HL-60 (18), and Jurkat (data not shown, see *Materials and Methods*). In each case the products were long, indicating that many repeats were synthesized. These data suggest that there may be important biochemical differences between the human and mouse telomerase preparations. These differences may be in the telomerase itself or in other components in the preparation. With the knowledge that mouse cell extracts synthesize very short products *in vitro*, the identification of telomerase activity in organisms where it has not previously been found may now be possible.

The difference in the length of the human and mouse telomerase products could be due to a difference in the processivity of the two enzymes. To determine whether the long products synthesized by the human enzyme are made in a processive or distributive manner, a primer challenge experiment was performed (7). A telomerase reaction was carried out for only 3 min with human 293 cell extract and [α-^{32}P]dGTP. After this initial pulse with [α-^{32}P]dGTP, part of the sample was diluted 30-fold into a mixture containing unlabeled dGTP and an excess of competitor $(TTAGGG)_3$ primer and the reaction was allowed to continue for a total of 9, 18, or 36 min (Fig. 3*A*). The products continued to increase

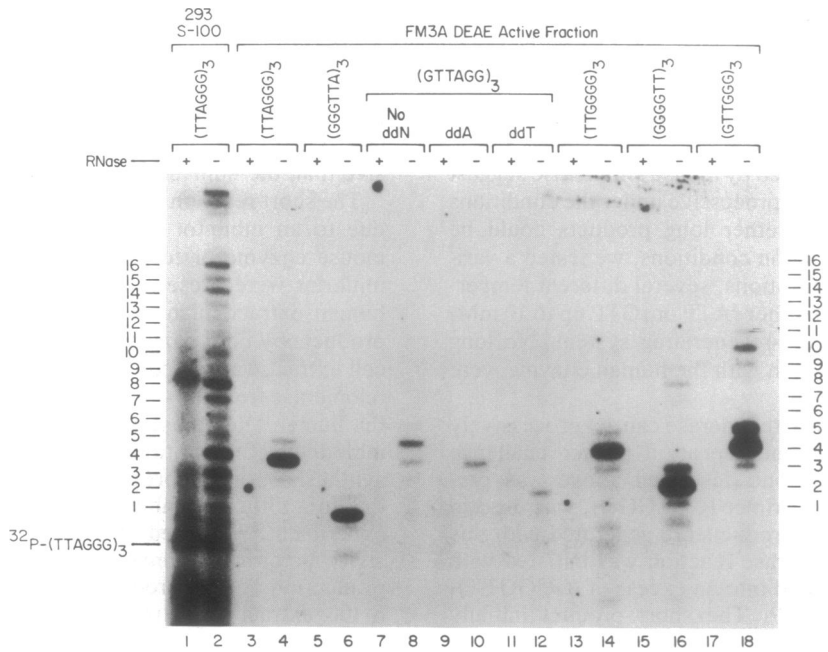

FIG. 2. Pausing pattern of mouse telomerase. The FM3A DEAE fraction was assayed for telomerase activity as in Fig. 1 with different telomeric oligonucleotides as primers. Lanes 1 and 2, human 293 cell S-100 extract, $(TTAGGG)_3$; lanes 3–18, FM3A DEAE fraction; lanes 3 and 4, $(TTAGGG)_3$; lanes 5 and 6, $(GGGTTA)_3$; lanes 7–12, $(GTTAGG)_3$; lanes 13 and 14, $(TTGGGG)_3$; lanes 15 and 16, $(GGGGTT)_3$; lanes 17 and 18, $(GTTGGG)_3$; lanes 9 and 10, 0.5 mM ddATP was used instead of dATP; in lanes 11 and 12, 0.5 mM ddTTP was used instead of dTTP. The position of a ^{32}P-labeled $(TTAGGG)_3$ is indicated. The numbers at left and right indicate the position of the additional nucleotides added to the input 18-mer primer. +/−, with/without RNase pretreatment.

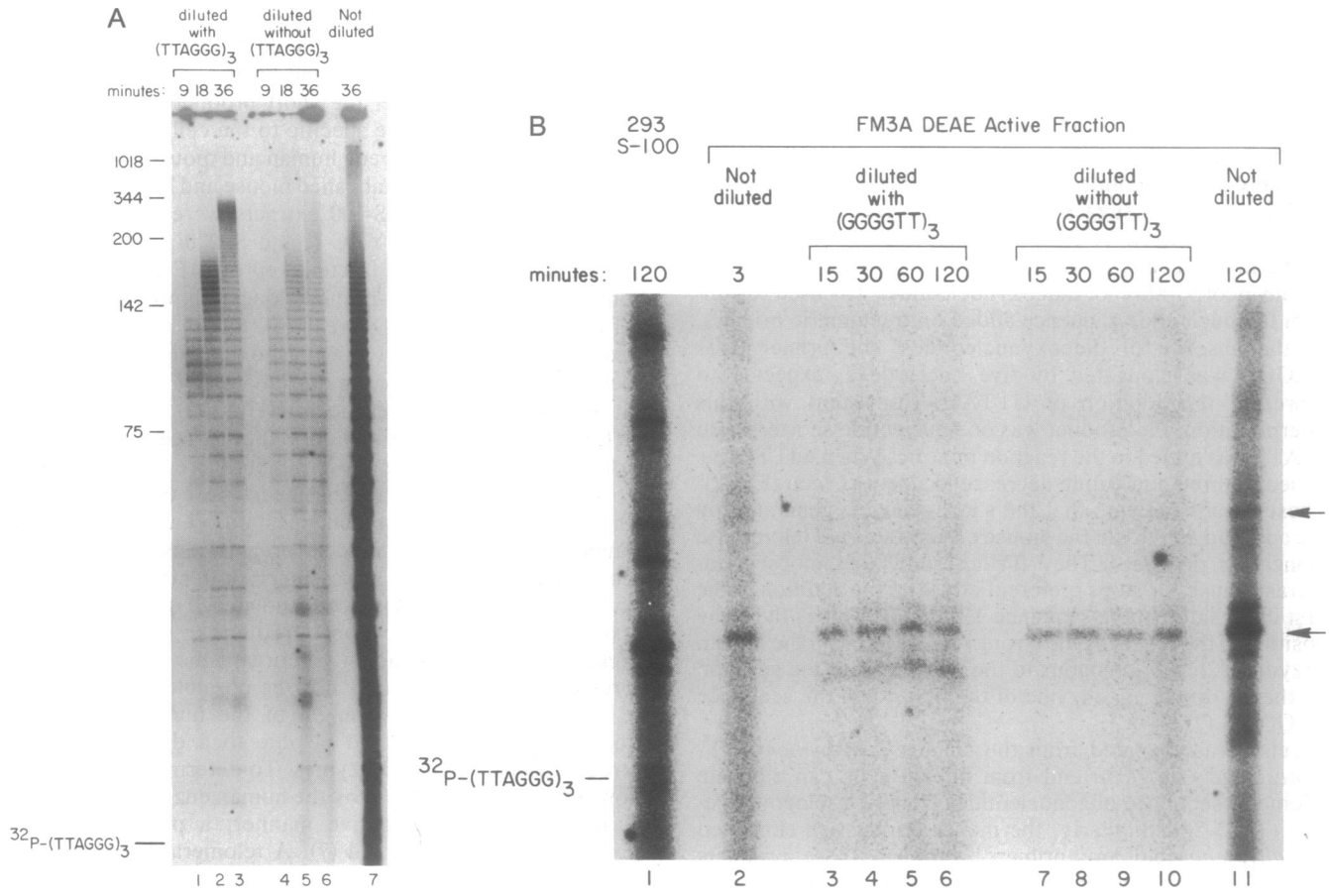

FIG. 3. Processivity of human and mouse telomerase. (*A*) Human telomerase primer-challenge experiment. S-100 extract from human 293 cells was assayed for processive telomerase activity as described in *Materials and Methods*. The reaction mixture was diluted after 3 min into unlabeled dGTP with excess primer (lanes 1–3) or with no primer (lanes 4–6), and aliquots were terminated at 9, 18, and 36 min. Lane 7, undiluted reaction mixture terminated at 36 min. Position of a ^{32}P-labeled $(TTAGGG)_3$ is indicated. Numbers at left indicate product length in nucleotides. (*B*) Mouse telomerase primer-challenge experiment. Lane 1, 293 S-100 extract (20 μl) was assayed as in Fig. 1 for 120 min. FM3A DEAE fraction was assayed as described in *Materials and Methods*. The reaction mixture was diluted after 3 min into unlabeled dGTP with excess primer (lanes 3–6) or no primer (lanes 7–10) and terminated at 15, 30, 60, and 120 min. Lanes 2 and 11, undiluted reaction mixture terminated at 3 min and 120 min, respectively. Arrows indicate the positions of the reaction products. The position of a ^{32}P-labeled $(TTAGGG)_3$ is indicated. The exposure shown is a PhosphorAutoradiograph (Fujix BAS 2000 BioImaging Analyzer) photograph from an HG printer (Fuji).

in length after dilution into unlabeled dGTP, indicating that the enzyme is processive *in vitro*.

The short products generated by mouse telomerase suggest that the enzyme is not highly processive under the conditions we used. To determine whether long products could be generated under other reaction conditions, we tested a variety of salts, $MgCl_2$ concentrations, several different temperatures, and the addition of either rATP or rGTP up to 10 mM, with and without an energy-regenerating system. No long products similar to those seen with the human enzyme were observed (data not shown).

To test whether the mouse telomerase can act processively to synthesize more than one repeat, a primer challenge experiment similar to the one described above was performed. The *Tetrahymena* primer $(GTTGGG)_3$ was used in the reactions, since longer products are generated with this oligonucleotide. The telomerase reaction was initiated with [α-^{32}P]dGTP and then diluted into an excess of $(GGGGTT)_3$ primer and unlabeled dGTP. The short product initially synthesized was not chased into higher repeats after 15, 30, 60, or 120 min of total reaction time (Fig. 3*B*). This suggests that the mouse telomerase is not processive. Because the low level of radioactive dGTP incorporated in the 3-min pulse, the synthesis of a second repeat without dissociation cannot be ruled out. However, it is clear from both the length of the products and the ratio of the first repeat to the second in the products of the human and mouse enzymes (Fig. 2, lanes 2 and 4) that the mouse telomerase is significantly less processive than the human enzyme.

The short reaction products in the mouse extracts could be due to an inhibitor which affects the processivity of the mouse enzyme or to a lack of a processivity factor. If an inhibitor were present, mixing the mouse extract with the human extract might result in the synthesis of only short products by both enzymes. To test this, 10 μl of human 293 cell extract was mixed with 1, 3, 5, or 10 μl of active mouse telomerase from the 0.2 M NaCl DEAE eluate. The ability of the human telomerase to synthesize long products was not inhibited by the addition of mouse extract (Fig. 4*A*). Thus the synthesis of only short products in the mouse extracts is not due to a diffusible inhibitor in the extract. In an analogous experiment, we tested whether there is a diffusible processivity factor in the human extracts which might stimulate the production of long products by the mouse enzyme. Human S-100 extract was treated with micrococcal nuclease to destroy telomerase activity. After inactivation of the micrococcal nuclease with EGTA, the extract was mixed with FM3A DEAE fraction. The addition of the inactivated human extract to active FM3A telomerase had no effect on the length of the mouse reaction products (Fig. 4*B*). A putative diffusible processivity factor may be present in the human extracts, but the species differences or reaction conditions used may

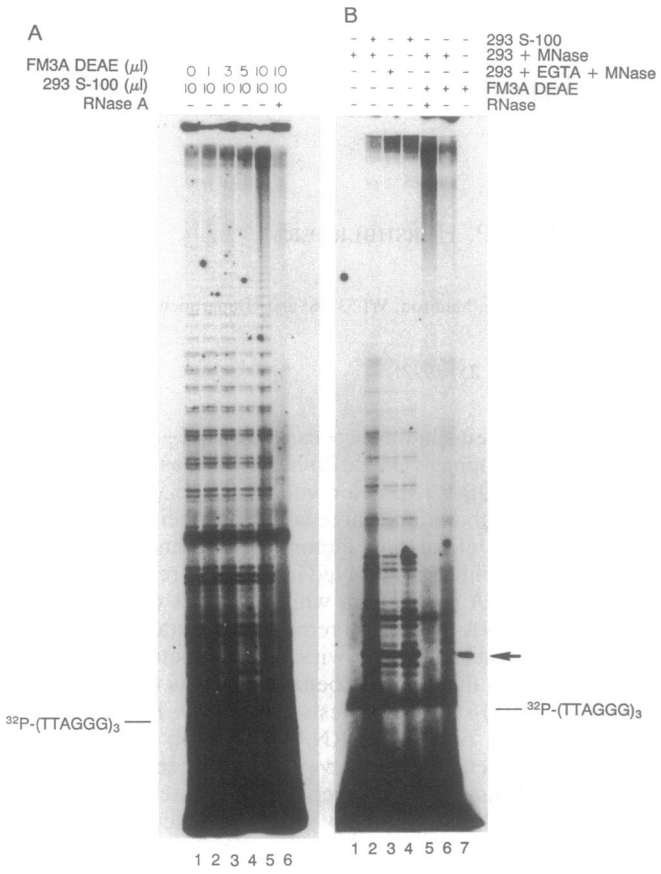

FIG. 4. Mixing of human and mouse telomerase extracts. (*A*) Ten microliters of 293 S-100 extract was mixed with 0 (lane 1), 1 μl (lane 2), 3 μl (lane 3), 5 μl (lane 4), or 10 μl (lanes 5 and 6) of FM3A DEAE fraction and assayed for telomerase activity as in Fig. 1. The position of a ^{32}P-labeled $(TTAGGG)_3$ is indicated. +/−, with/without RNase A pretreatment. (*B*) Samples of 293 S-100 extract alone (lane 4) or treated with inactivated micrococcal nuclease (MNase, lane 3) or active MNase (lane 1) were assayed for telomerase activity. A portion of the sample treated with MNase was then inactivated by addition of EGTA and mixed with fresh 293 S-100 (lane 2) or with FM3A DEAE fraction (lanes 5 and 6). The sample in lane 5 was pretreated with RNase A before assay for telomerase activity. Lane 7, FM3A DEAE extract alone. The position of a ^{32}P-labeled $(TTAGGG)_3$ is indicated. Arrow indicates the position of the FM3A reaction product.

have prevented stimulation of the mouse enzyme. Finally, mixing of inactive mouse DEAE fractions (0.1, 0.3, and 0.4 M NaCl eluates) with the active 0.2 M NaCl fraction did not result in the generation of long products by the mouse enzyme (data not shown). If hybrid mouse–human cell lines containing only one human chromosome are tested for the ability to synthesize long telomerase products, it may be possible to identify a human processivity factor and assign it or the human telomerase to a specific chromosome.

Telomerase preparations from five different immortal human cell lines all generated long products *in vitro*, whereas telomerase from four different mouse cell lines synthesized very short products. Although this is not an exhaustive study, it suggests that there is a species or biochemical difference in the processivity of human and mouse telomerase enzymes. Since *Mus musculus* has been characterized as having "ultra-long telomeres" in vivo (20), it is interesting that the 'ultra-short' telomerase products generated *in vitro* are derived from this species. Human telomeres have shorter stretches of $(TTAGGG)_3$ than mouse (30, 31), and yet human telomerase activity is more processive *in vitro*. A similar discrepancy between the *in vivo* and *in vitro* processivity is found with *Tetrahymena* telomerase (7, 8). These observations suggest that factors other than telomerase may be involved in the *in vivo* regulation of telomere length. The evidence that telomeres may play a role in cellular aging and immortalization (15, 16, 18) illustrates the importance of identifying all of the factors which regulate telomere length. The biochemical differences between the human and mouse telomerase may aid in the identification of factors which regulate both telomerase activity and telomere length. Identification of mouse telomerase will also allow the use of transgenic mice to test the roles of telomere length and telomerase *in vivo*.

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