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Telomerase activity in germline and embryonic cells of *Xenopus*

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Telomerase is a ribonucleoprotein which synthesizes telomere repeats onto chromosome ends. Telomerase activity is involved in telomere length maintenance. We used *Xenopus laevis* as a model system to study the expression of telomerase activity in germline cells and during early development. We identified a non-processive telomerase activity in manually dissected nuclei of *Xenopus* stage VI oocytes. Telomerase activity was detected throughout oogenesis and embryogenesis. Telomerase was active in both S and M phase cell cycle extracts, suggesting that telomerase activity is not regulated with chromosomal DNA replication.

Key words: cell cycle/embryogenesis/germline/oogenesis/telomerase

Introduction

Telomeres are essential chromosomal components required for chromosome stability and length maintenance. Telomere sequences are conserved among eukaryotes; they consist of tandem repeats of short G-rich sequences. The number of repeats on any given chromosome is not fixed and thus telomeres appear as heterogeneous or 'fuzzy' bands on Southern blots (reviewed in Blackburn, 1991). Conventional DNA replication cannot complete the replication of linear chromosome ends. To balance chromosomal shortening, telomeric repeat sequences are added *de novo* onto chromosome ends by the enzyme telomerase (reviewed in Blackburn, 1991). Telomerase is a ribonucleoprotein, and its RNA component specifies the addition of specific telomeric sequence repeats (Greider and Blackburn, 1989; Shippen-Lentz and Blackburn, 1990; Yu *et al.*, 1990). Telomerase activity was first identified in the ciliate, *Tetrahymena* (Greider and Blackburn, 1985). Telomerase has since been identified in immortalized human and mouse cell lines (Morin, 1989; Counter *et al.*, 1992; Prowse *et al.*, 1993).

Although telomere length is maintained in single cell eukaryotes, such as *Tetrahymena* and yeast, telomere length regulation appears to be very different in mammalian cells. In primary human somatic cells, telomeres shorten with each cell division *in vivo* and *in vitro*. Tissues from older donors have shorter telomeres than those of young donors (Harley *et al.*, 1990; Hastie *et al.*, 1990; Allsopp *et al.*, 1992; Vaziri *et al.*, 1993). In contrast, human sperm telomeres are longer than those of somatic cells, and are maintained or even lengthened with increased donor age (Harley *et al.*, 1990; Vaziri *et al.*, 1993). The regulation of telomerase activity appears to be involved in these telomere length differences.

Established immortalized human cell lines often have short telomeres and telomere length does not decrease during cell growth. The regulation of telomerase activity is different before and after the establishment of immortalized human cells. Telomerase activity is not detectable in primary human embryonic kidney (HEK) and primary fibroblast cell extracts. After transfection of the HEK cells with SV40, telomere length decreased and telomerase activity was not detected. After ~100 cell divisions the culture went through crisis and most of the cells died. Those clones which survived crisis had short stable telomere length and detectable telomerase activity. This result suggested that clones which survived crisis had to up-regulate or reactivate telomerase to maintain their telomere length. Inhibiting telomerase may thus inhibit the growth of these immortalized cells (Counter *et al.*, 1992).

The long telomeres in human spermatozoa suggest that telomerase may be active in germline tissue to maintain telomeres for future generations. To investigate the apparent tissue specificity and developmental regulation of telomerase activity, we have begun studies in both mice and the toad *Xenopus laevis*. As we predicted, telomerase activity was present in mouse testes (K.R.Prowse and C.W.Greider, in preparation). To test whether telomerase is expressed in ovarian tissue or oocytes, we chose *X.laevis* as a model system. During oogenesis, large amounts of protein and RNA are synthesized as a maternal stock for early development (Dumont, 1972). Cardenas *et al.* (1993) reported that *Xenopus* ovaries contain a very abundant telomere binding protein and they suggest that this factor is stock-piled in oocytes for rapid early embryonic cell divisions. We therefore reasoned that since telomerase may be present in germline cells, *Xenopus* stage VI oocytes may provide a rich source of telomerase. We report here on the identification and characterization of *Xenopus* telomerase in germline cells.

In addition to being a good source of telomerase in ovarian tissue, *Xenopus* is also excellent for studies of early embryonic development. Since large numbers of fertilized eggs are available and the development is external, embryos at different stages can be easily obtained. Furthermore, the development of an *in vitro* cell cycle system, that mimics *in vivo* cell cycle events, facilitates the study of telomerase regulation during the cell cycle of *Xenopus* early embryogenesis (Lohka and Masui, 1983; Blow and Laskey, 1986; Hutchison *et al.*, 1987; Murray and Kirschner, 1989). We have taken advantage of the large body of knowledge on early development in *Xenopus* to study the expression of telomerase during early developmental stages.

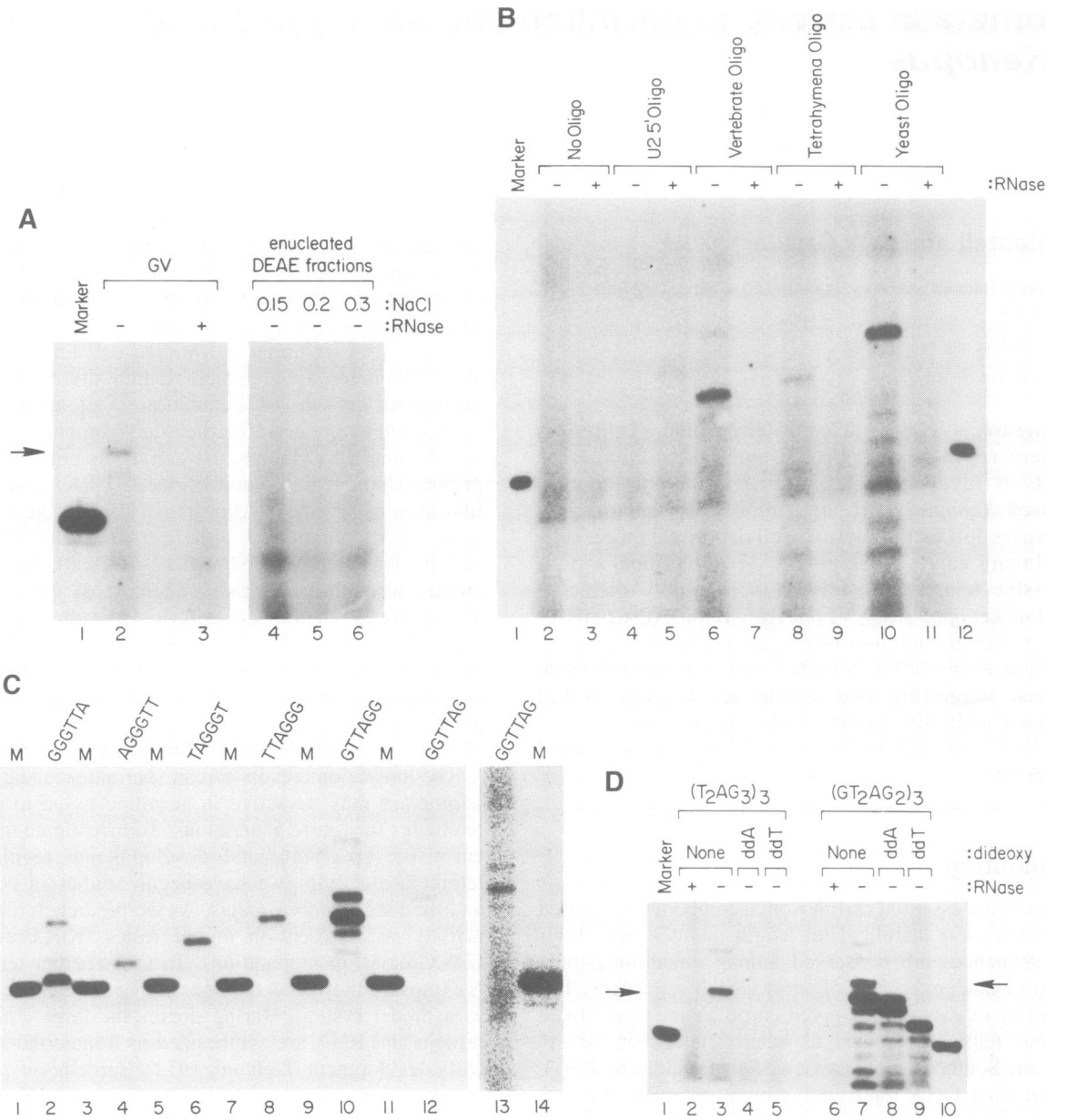

Fig. 1. Identification of *Xenopus* telomerase. (**A**) Telomere elongation activity in stage VI oocyte extracts. Lane 1, a marker [α-^{32}P]dideoxyATP end-labeled oligonucleotide $(TTAGGG)_3$. Lanes 2 and 3, the nuclear lysate of 40% of a stage VI oocyte. Lanes 4–6, the DEAE-purified fractions of the enucleated extracts. The numbers in lanes 4–6 indicate the NaCl concentration in the different DEAE elution buffers. The arrow indicates the four nucleotides elongation product. (**B**) Primer specificity. Lanes 2 and 3, a 0.2 M NaCl fraction of total oocyte extracts after DEAE column purification was assayed for telomerase activity in the absence of oligonucleotides. Lanes 4–11, telomerase assayed in the presence of different oligonucleotides. Each oligonucleotide is assayed with and without pretreatment with RNase. Lanes 1 and 12, the [α-^{32}P]dideoxyATP end-labeled marker $(TTAGGG)_3$. Lanes 4 and 5, a non-telomeric oligonucleotide, GGCCGAGAAGCGAT; lanes 6 and 7, vertebrate telomere sequence, $(TTAGGG)_3$; lanes 8 and 9, *Tetrahymena* telomere sequence, $(TTGGGG)_3$; lanes 10 and 11, a sequence corresponding to yeast telomeres, $(TGTGTGGG)_2TG$. (**C**) Elongation patterns using permuted sequence $(TTAGGG)_3$ primers. The 0.2 M NaCl fraction of total oocyte extracts after DEAE column purification was assayed for telomerase activity with different primers. Lane 2, $(GGGTTA)_3$; lane 4, $(AGGGTT)_3$; lane 6, $(TAGGGT)_3$; lane 8, $(TTAGGG)_3$; lane 10, $(GTTAGG)_3$; lanes 12 and 13, $(GGTTAG)_3$. Lanes 1, 3, 5, 7, 9, 11 and 14, [α-^{32}P]dideoxyATP end-labeled input primer. Lanes 13 and 14 are the inverted, darker exposure of lanes 11 and 12 by Fuji PhosphoImager. (**D**) Effects of dideoxynucleotides on *Xenopus* telomerase. Telomerase reactions were carried out as described in Materials and methods. In lanes 4, 5, 8 and 9, dideoxynucleotides were substituted for the deoxynucleotide triphosphates. Lanes 2–5, input primer $(TTAGGG)_3$, lanes 6–9, input primer $(GTTAGG)_3$. Lanes 2, 3, 6 and 7, no dideoxynucleotides added; lanes 4 and 8, dideoxyATP added; lanes 5 and 9, dideoxyTTP added. Lanes 1 and 10, [α-^{32}P]dideoxyATP end-labeled $(TTAGGG)_3$. Arrows indicate the position of the **G** pause of telomerase elongation products on 8% sequencing gels. +/–, plus or minus RNase pretreatment of the extracts.

Results

Identification of telomerase activity in stage VI oocyte nuclei

Since telomerase is involved in the synthesis of telomere repeats, telomerase activity is expected to be present primarily in the nucleus. We manually dissected nuclei, or germinal vesicles (GV), from stage VI oocytes and prepared nuclear lysate. Although *Xenopus* telomeres have not been cloned and sequenced, *in situ* hybridization and Southern blot analysis suggest that they consist of the characteristic vertebrate TTAGGG repeats (Allshire *et al.*,

1988; Meyne *et al.*, 1989). Therefore, the oligonucleotide $(TTAGGG)_3$ was used as a primer for telomerase assays. Two predominant elongation products, four and seven nucleotides longer than the input $(TTAGGG)_3$ primer, were detected in the nuclear lysate (Figure 1A, lane 2). This primer elongation activity was abolished by RNase pretreatment of the extracts (Figure 1A, lane 3). There was a large amount of background labeling in the enucleated extract (data not shown). DEAE–agarose chromatography has been used previously for purification of telomerase (Greider and Blackburn, 1987; Prowse *et al.*, 1993). We therefore used a DEAE column to fractionate the enucleated extract. No primer elongation activity was detected in the enucleated fractions (Figure 1A, lanes 4–6).

Both the ciliate and human telomerases are processive *in vitro*. However, mouse telomerase is less processive and generates one predominant product from the addition of one TTAGGG repeat (Morin, 1989; Greider, 1991; Prowse *et al.*, 1993). To determine whether the *Xenopus* primer elongation activity was due to telomerase, we examined the primer specificity of elongation in oocyte extracts. Telomerase from all other organisms specifically elongates only GT-rich telomere-like primers (Greider and Blackburn, 1987; Zahler and Prescott, 1988; Morin, 1989; Prowse *et al.*, 1993). No elongation products were detected in the absence of primers or in the presence of non-G-rich primers (Figure 1B, lanes 2 and 4). However, telomeric sequence primers were elongated (Figure 1B, lanes 6, 8 and 10). These properties are similar to those of the non-processive telomerase activity identified in mouse cells (Prowse *et al.*, 1993). When the *Tetrahymena* telomeric primer $(TTGGGG)_3$ was used, the intensity of the elongation product was reduced compared with assays using the primer $(TTAGGG)_3$ (Figure 1B, lane 8). Greater levels of elongation products were generated with the oligonucleotide corresponding to the yeast telomeric sequence $(TGTGTGGG)_2TG$ (Figure 1B, lane 10). These differences in primer utilization may be due to the differences in binding affinity of telomerase to the different substrates.

Tetrahymena telomerase pauses after copying to the end of the internal RNA template. For the addition of the first telomere repeat, different numbers of nucleotides are added onto primers with different 3′ ends and the correct next nucleotide is always added (Greider and Blackburn, 1987; Greider, 1991). If the elongation products generated in *Xenopus* oocyte extract were made by telomerase, we expect the length of the predominant products to be dependent on the 3′ sequence of the input primer oligonucleotide. We assayed telomerase activity using primer oligonucleotides with permutations of the sequence $(TTAGGG)_3$ (Figure 1C). Predominant products with the addition of 1, 2, 3 and 4 nucleotides were generated with oligonucleotide primers $(GGGTTA)_3$, $(AGGGTT)_3$, $(TAGGGT)_3$ and $(TTAGGG)_3$, respectively (Figure 1C, lanes 2, 4, 6 and 8). Using the oligonucleotides $(GTTAGG)_3$ and $(GGTTAG)_3$ as primers, the darkest bands were one nucleotide shorter than expected, although the expected full-length elongation products were also present (Figure 1C, lanes 10 and 12). In addition, elongation of $(GGTTAG)_3$ generated lower amounts of products than other primers, although a darker exposure showed that this primer was elongated as expected (Figure 1C, lanes 12 and 13). The product length generated with permutated

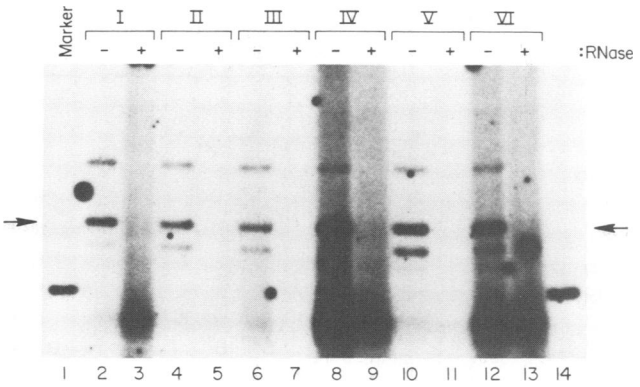

Fig. 2. Telomerase is expressed throughout *Xenopus* oogenesis. Lanes 2–7, telomerase activity assayed in the S100 extracts of stage I–III oocytes. Lanes 8–13, telomerase activity assay in 0.2 M NaCl DEAE fractions of stage IV–VI total oocyte extracts. A high background labeling, possibly due to the contamination of yolk accumulated in late stage oocytes, was seen in the S100 extracts of stage IV–VI oocytes (data not shown). Thus, to see telomerase activity in the late stage oocytes, these extracts were purified over a column. Most of the telomerase activity was recovered in the 0.2 M NaCl fraction off a DEAE column (data not shown). Telomerase reactions were carried out with untreated fractions or fractions which had been pretreated with RNase, as indicated. Extracts from equivalent cell numbers were assayed in each lane. Lanes 1 and 14, [α-^{32}P]dideoxyATP end-labeled $(TTAGGG)_3$. Arrows indicate the position of the predominant telomerase elongation products on an 8% sequencing gel.

sequence primers indicated that the products were synthesized by telomerase. In addition to the predominant elongation products, a product three nucleotides longer than the primary band was seen for the elongation of each telomeric primer (Figure 1B and C). Such a band was not found with the mouse telomerase (Prowse *et al.*, 1993). The size and nuclease sensitivity suggest that this band represents a second pause of *Xenopus* telomerase elongation at the next T residue in the sequence **GGGTTA**.

To further test if the sequence TTAGGG was added onto the input telomeric primers, dideoxynucleotides were substituted for deoxynucleotides to terminate the elongation reaction at specific positions (Figure 1D and data not shown). Using the oligonucleotide primer $(GTTAGG)_3$, elongation products with the addition of up to four nucleotides were generated in the presence of ddATP, while elongation products with the addition of only two nucleotides were generated in the presence of ddTTP (Figure 1D, lanes 8 and 9). This is consistent with the addition of the sequence GTTAG onto the $(GTTAGG)_3$ primer. In the presence of either ddATP or ddTTP, no [α-^{32}P]dGTP was incorporated into the input primer $(TTAGGG)_3$ (Figure 1D, lanes 4 and 5), indicating that TTP and dATP were incorporated before [α-^{32}P]dGTP. This is consistent with the addition of TTAG onto the $(TTAGGG)_3$ primer. These results confirmed that *Xenopus* oocyte extracts contain telomerase activity which completes the last repeat GGTTAG of input telomeric primers and terminates at the first G of TTA**GGG** or the following T of TTA**GGGT**.

Detection of telomerase activity throughout Xenopus oogenesis

To initiate our investigation of telomerase regulation, we assayed telomerase activity at each stage of oogenesis.

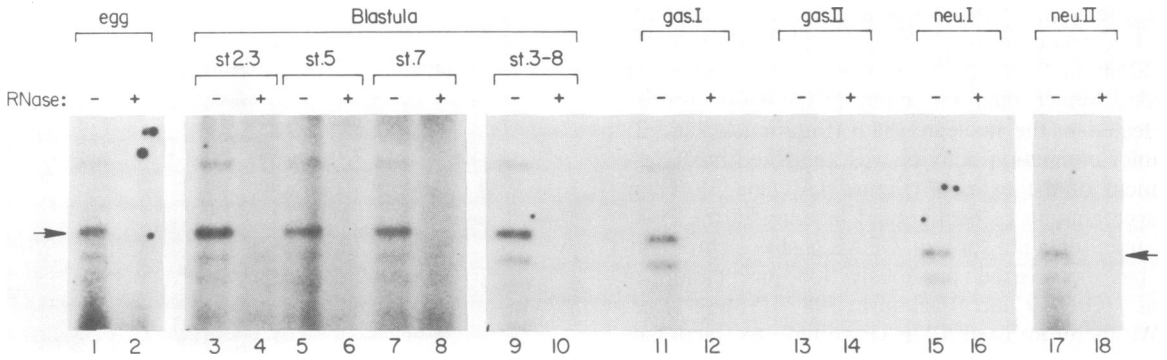

Fig. 3. Characterization of telomerase activity throughout *Xenopus* embryogenesis. Telomerase activity assays were carried out with 0.2 M NaCl DEAE fractions of total embryonic extracts at different stages in the presence or absence of RNase pretreatment. Lanes 1 and 2, egg; lanes 3–10, stage 2 to stage 8 blastula; lanes 11–14, early and late stage of gastrula; lanes 15–18, early and late stage of neurula. Extracts from equivalent cell numbers were assayed in each lane. Telomerase elongation products were detected in each extract, although lower amounts of products were seen from the assay of the extract prepared from late stage gastrula. Arrows indicate the position of the predominant telomerase elongation products on 8% sequencing gels.

Approximately 200 oocytes from stages I to VI were visually staged and S100 extracts were prepared (see Materials and methods). Telomerase from the S100 extracts of stage IV–VI oocytes was purified over a DEAE–agarose column to remove background labeling in the crude extracts (Prowse *et al.*, 1993). Most of the *Xenopus* telomerase activity was recovered in a 0.2 M NaCl wash from a DEAE–agarose column (data not shown). Extracts representing all stages of oocyte development had telomerase activity (Figure 2).

Telomerase activity is present throughout Xenopus embryogenesis

To determine whether telomerase was active during *Xenopus* early development, we obtained fertilized eggs and allowed development to occur. Embryogenesis in *Xenopus* is a relatively rapid process which is complete within 2 days at room temperature. Embryos at different stages were identified based on their morphology (Nieuwkoop and Faber, 1967; Danilchick and Peng, 1991). S100 extracts were prepared from embryos at different developmental stages and assayed for telomerase activity using the standard assay. Telomerase activity was detected in eggs and at all stages of embryogenesis, including blastula, gastrula and neurula, although the amount of the telomere elongation products produced by the late-stage gastrula extract was lower than at other stages (Figure 3).

Expression of telomerase activity in both the M and S phases of the cell cycle during early Xenopus embryogenesis

To examine whether telomerase activity is regulated during early embryonic cell cycles, S and M phase-specific extracts were prepared from *Xenopus* eggs (Smythe and Newport, 1991). In *Xenopus*, the first 12 cell divisions after fertilization oscillate between S and M phases in a synchronized manner with no measurable G_1 and G_2 phases (Graham and Morgan, 1966). When eggs are disrupted in the presence of EGTA, the extracts have high maturation-promoting factor (MPF) activity and are able to disassemble exogenously added nuclei, essentially mimicking cells in meiosis. However, when the eggs are broken in the absence of EGTA, calcium is released from internal stores and the extracts exhibit characteristics of cells in S phase (Newport, 1987; Wilson and Newport, 1988; Smythe and Newport, 1991). S phase-specific extracts can assemble nuclei and have low levels of MPF activity.

We prepared S and M phase extracts from *Xenopus* eggs (see Materials and methods). For the S phase extract, cycloheximide was added to the buffer to prevent any new synthesis of cyclin which may induce MPF activity in the extract (Smythe and Newport, 1991). Telomerase activity was detected in both S and M phase extracts that were purified by either DEAE–agarose chromatography or $(NH_4)_2SO_4$ fractionation (Figure 4A and data not shown). *Xenopus* telomerase activity is present in the supernatant of 15% $(NH_4)_2SO_4$ precipitation (data not shown). Telomerase activity was also detected in both the crude S and M phase extracts after simple dilution without further purification (data not shown).

As a control for the specificity of the S and M phase extracts, MPF activity was tested in these extracts. A synthetic polypeptide substrate, specific for $p34^{cdc2}$ protein kinase, was used to assay for MPF activity (Marshak *et al.*, 1991). As expected, the M phase extract had a higher MPF activity than the S phase extract (Figure 4B). In addition, a nuclear assembly assay was performed with these extracts (Smythe and Newport, 1991). When *Xenopus* sperm chromatin was added to the S phase extract, the sperm chromatin decondensed and formed nuclei; in contrast, the morphology of the sperm chromatin remained the same when the sperm chromatin was incubated with the M phase extract (data not shown).

Identification of telomerase activity in mature sperm cells

To test whether telomerase activity was present in spermatozoa, extracts of *Xenopus* testes were assayed for telomerase activity. Telomerase activity was detected in total testes extracts (Figure 5, lane 2). *Xenopus* testes are composed of primary and secondary spermatocytes, spermatids, mature spermatozoa and supporting somatic cells, including Sertoli and red blood cells. To determine if telomerase activity is present in sperm cells, mature spermatozoa were separated from the remaining testes,

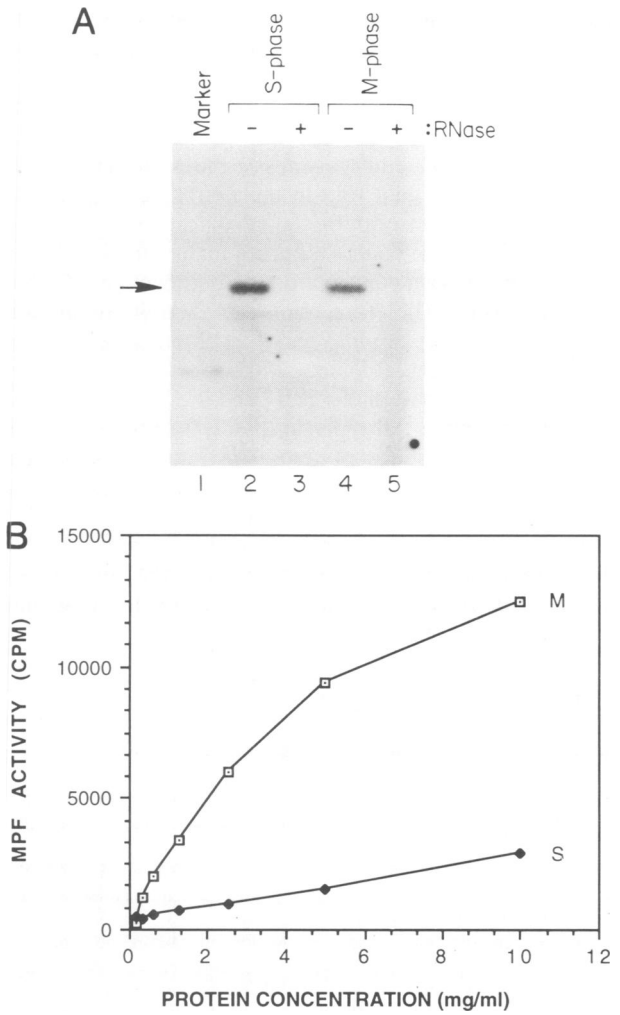

Fig. 4. (**A**) Characterization of telomerase activity in cell cycle-specific extracts. Telomerase assays were carried out with (+) and without (–) pretreatment of the extract with RNase. Lanes 2 and 3, telomerase activity assayed in the DEAE column-purified S phase extract. Lanes 4 and 5, telomerase activity assayed in the DEAE column-purified M phase extract. Extracts from equivalent cell numbers were assayed in each lane. Lane 1, [α-^{32}P]dideoxyATP end-labeled oligonucleotide $(TTAGGG)_3$. The arrow indicates the position of the predominant telomerase elongation products on an 8% sequencing gel. (**B**) Characterization of MPF activity in cell cycle extracts. MPF activity was measured as [γ-^{32}P]ATP incorporation into the p34^{cdc2} protein kinase-specific polypeptide substrate. A series of dilutions were made for both M and S phase extracts. (⊡) M-phase extract; (◆) S-phase extract.

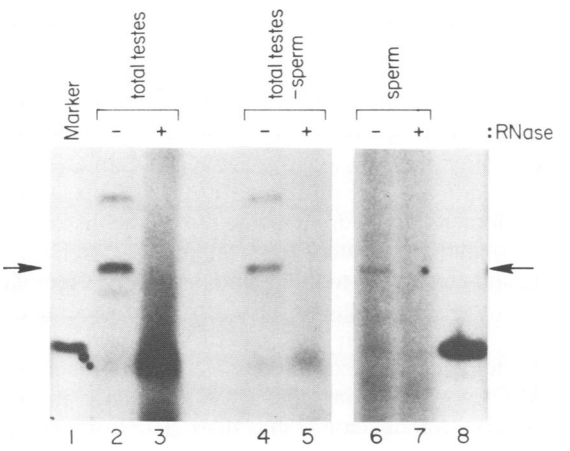

Fig. 5. Telomerase is active in *Xenopus* spermatozoa. Telomerase activity was assayed in 0.2 M DEAE fractions of each extract. Telomerase assays were carried out with (+) and without (–) pretreatment of the extract with RNase. Lanes 2 and 3, total testes extracts; lanes 4 and 5, the remaining testes after the removal of mature spermatozoa; lanes 6 and 7, 10^6 mature spermatozoa in each lane. Lanes 1–5, 1 day exposure. Lanes 6–8, 7 day exposure. Lanes 1 and 8, [α-^{32}P]dideoxyATP end-labeled oligonucleotide $(TTAGGG)_3$. Arrows indicate the position of the predominant telomerase elongation products on an 8% sequencing gel.

washed and pelleted five times and tested for telomerase activity. No activity was present in the final wash. However, a low level of telomerase activity was detected in extracts prepared from ~10^6 mature sperm cells (Figure 5, lane 6). To determine if this low level of activity was due to telomerase bound to the outside of the spermatozoa, the spermatozoa were treated with micrococcal nuclease after washing. The micrococcal nuclease was then inactivated by addition of EGTA, and the spermatozoa were washed three times and lysed to prepare the S100 extract. A low level of activity was still detected in the sperm S100 extract, although no telomerase activity was detected in the final wash (data not shown). Telomerase activity was present also in the extract prepared from testes after the removal of most mature spermatozoa (Figure 5, lane 4). The telomerase activity detected in this extract may be from somatic cells, or from immature spermatocytes, spermatids and the remaining mature spermatozoa.

Discussion

We have identified a non-processive telomerase activity in the nuclei of *Xenopus* stage VI oocytes. The detection of *Xenopus* telomerase activity throughout oogenesis, embryogenesis and in mature spermatozoa, suggests that telomerase may play a role in germline cells and during early development. The presence of telomerase activity at different stages of the cell cycle implies that telomerase may not be associated solely with chromosomal DNA replication.

In vitro, telomerase activity identified in both human cell lines and *Tetrahymena* is processive, although telomerase activity from mouse cells and *Xenopus* cells is non-processive. The difference in processivity in different organisms may be due to *in vitro* conditions, such as specific factors that are associated with telomerase during isolation, or may reflect true differences in the enzymes. *Xenopus* telomerase non-processively elongated permuted $(TTAGGG)_3$ oligonucleotides and primers corresponding to telomere sequences of different species (Figure 1B and C). Interestingly, the amounts of the elongation products generated by *Xenopus* telomerase onto these oligonucleotide primers correlate with the *in vivo* stability of linear plasmids containing these sequences. *In vivo*, linear DNA molecules containing human or yeast telomeres can be stably replicated in either *Xenopus* eggs or oocytes, while those containing *Tetrahymena* telomeres are degraded (Schmid *et al.*, 1991; Weber *et al.*, 1993). *In vitro*, *Xenopus* telomerase generated more elongation products on both the vertebrate $(TTAGGG)_3$ primers and the yeast $(TGTGTGGG)_2TG$ primer than on the *Tetrahymena* $(TTGGGG)_3$ primer (Figure 1B). This difference may be

due to the binding or alignment between the primers and the telomerase RNA.

Two lines of evidence suggest that *Xenopus* telomerase RNA may contain the sequence 5′-CUAACCCX- within the RNA template. First, most of the elongation of telomeric oligonucleotides stops at the first G of GGTTAG after the addition of one repeat of TTAGGG. This termination of telomere elongation may be due to the dissociation of the elongation products from telomerase after primer binding and elongation to the 5′ end of the RNA template. Second, very few elongation products were generated by *Xenopus* telomerase on the $(GGTTAG)_3$ primer. Since *Xenopus* telomerase is non-processive, if the sequence 5′-CUAACCCX- serves as the RNA template, most of $(GGTTAG)_3$ oligonucleotide may dissociate from telomerase without elongation because the primer may align entirely within the RNA template, and not be a substrate for further elongation.

The primers $(GTTAGG)_3$ and $(GGTTAG)_3$ generated a primary elongation product one nucleotide shorter than the expected product. This predominant band may be due to specific cleavage of the full-length product of the permuted primers. A primer-specific intrinsic cleavage activity has been characterized for the *Tetrahymena* telomerase enzyme (Collins and Greider, 1993). Only those primers having a specific permutation of the telomeric repeat d(GGGTTG) are cleaved. Preliminary results indicate that a primer-specific cleavage activity is also associated with the *Xenopus* telomerase (data not shown).

The detection of telomerase activity in *Xenopus* oocytes and mature spermatozoa confirms the prediction that telomerase activity is present in germline cells. The role of the telomerase activity in mature spermatozoa is not clear. Interestingly, telomerase activity from only 40% of a single stage VI oocyte nucleus was clearly visualized, implying that telomerase is highly abundant or highly active in this tissue (Figure 2). Beginning at stage II of *Xenopus* oogenesis, large amounts of RNA and protein are synthesized and stored as the maternal stock (Taylor and Smith, 1985; Davidson, 1986). Since telomerase is present in stage I oocytes, activity in oocytes cannot be derived solely from protein synthesis in the later stages of oocyte development.

Telomerase activity was detected in whole embryos at all stages of development up to neurulation. Telomerase activity in these later stages of embryogenesis may represent the expression of telomerase from the zygotic genome. During gastrulation and neurulation, primary differentiation takes place. Although cell differentiation has been proposed to down-regulate telomerase activity (Harley, 1991; Harley *et al.*, 1992), *Xenopus* telomerase activity was detected in whole embryonic extracts after differentiation. However, our studies did not address whether specific tissues are expressing telomerase and other tissues lack telomerase activity at these stages.

Detection of telomerase activity in the oocyte nuclear lysate suggests that telomerase functions in the nucleus with DNA polymerases and primase to maintain telomere length. However, telomerase activity was also found at stages not associated with DNA replication, such as the M phase of the cell cycle, and in mature spermatozoa and in immature oocytes. This finding suggests that telomerase activity may not be cell cycle-regulated in *Xenopus*, or that the early embryonic stages of development have highly active telomerase not found at later stages. A detailed investigation of telomerase activity and telomere lengths in adult *Xenopus* will be necessary to address this issue.

During *Xenopus* embryogenesis, the maternal oocyte stores play a dominant role in the first 12 cell divisions before zygotic transcription takes place (Newport and Kirschner, 1982). In contrast, during mammalian embryogenesis, the maternal contribution is significant only for the first cell division. Therefore, telomerase activity regulation may differ between *Xenopus* early development and mammalian development. Telomerase appears to be inactive in human somatic tissues and reactivated in cancer cells. The ability of immortal cell types to divide indefinitely seems to require the presence of telomerase activity (Counter *et al.*, 1992, 1994). It is not yet clear whether this same regulation occurs in *Xenopus*. Although telomerase regulation may differ in some respects between *Xenopus* and mammalian cells, by understanding the differences and similarities, we will gain insights into the essential components of both systems.

Materials and methods

Isolation of Xenopus oocytes, eggs, embryos and spermatozoa

Female toads were sacrificed to obtain ovarian tissues. Oocytes were dissociated from ovary segments by digesting in a solution of 0.2% collagenase (type I, Sigma Chemical Co., St Louis, MO) in modified Barth's saline (MBS; which contains 88 mM NaCl, 1 mM KCl, 0.7 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mM HEPES, pH 7.8, 2.5 mM $NaHCO_3$) and rinsed extensively in MBS (Harland and Sive, 1993). Different stages of oocytes were separated under a dissecting microscope according to their size and morphology (Dumont, 1972; Smith *et al.*, 1991). Stage VI nuclei were obtained by manual dissection under a dissecting microscope (Evans and Kay, 1991).

To obtain eggs, female toads were primed with pregnant mare serum gonadotropin (PMSG, Sigma) and injected with human chorionic gonadotropin (HCG, Sigma) to induce ovulation. Eggs were squeezed from the toads into MBS, dejellied in a solution containing 2% cysteine (free base, Sigma) in 0.2×MBS, pH 7.8, and rinsed extensively with MBS (Murray, 1991). Embryos were obtained by natural mating of HCG-injected male toads and HCG-induced female toads (Nasco, protocol from the vendor), and staged as described (Nieuwkoop and Faber, 1967).

To obtain mature spermatozoa, testes were removed after male toads had been sacrificed, and were then rinsed and minced in extract preparation buffer [EPB; which contains 10 mM HEPES, pH 8.0, 3 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, protease inhibitors (PMSF, leupeptin, pepstatin A) and RNase inhibitor 10 U/ml RNasin (Promega)] in the presence of 0.2 M sucrose. Testes minced in EPB were spun in a clinical centrifuge at 170 *g* for 10 s, and mature spermatozoa were pelleted at 1350 *g* for 2 min. The mature sperm pellet was rinsed in EPB, pelleted at 1350 *g* for 2 min five times and homogenized in EPB in the presence of 0.05% lysolecithin (Sigma) (Smythe and Newport, 1991). To avoid contamination by telomerase that may reside on the surface of sperm cells, the spermatozoa were treated with micrococcal nuclease (Boehringer Mannheim), and then with EGTA to inactivate micrococcal nuclease. The treated spermatozoa were then washed three times and lysed to prepare S100 extract.

Cell extracts

Eggs were crushed and centrifuged in EPB under 100 000 *g* for 60 min. Glycerol and NaCl were then added to the S100 extracts to final concentrations of 10% and 0.1 M, respectively. Oocytes, embryos, testes and mature spermatozoa were homogenized in EPB to allow the breakage of nuclei, spun at 100 000 *g* and treated as described above. To purify telomerase activity, extracts were loaded onto a DEAE–agarose column equilibrated with EPB in the presence of 0.1 M NaCl and 10% glycerol. The column was washed in this buffer and eluted with two column

volumes of 0.2 M NaCl, and subsequently with 0.3 M NaCl, in EPB and 10% glycerol. Nuclear extracts of stage VI oocytes were prepared by placing dissected nuclei in EPB in the presence of 0.1 M NaCl and 10% glycerol and lysing by pipetting them through micropipette tips.

S and M phase-specific extracts were prepared according to Smythe and Newport (1991). In brief, eggs were centrifuged at 12 000 *g* for 30 min in a buffer containing 250 mM sucrose, 2.5 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 10 mM HEPES, pH 7.7, 50 μg/ml cycloheximide and 5 μg/ml cytochalasin for the S phase extract. Eggs were lysed in a buffer containing 240 mM Na β-glycerophosphate, pH 7.3, 60 mM EGTA, 45 mM $MgCl_2$, 1 mM DTT and 5 μg/ml cytochalasin for the M phase extract. The cytoplasmic extracts were withdrawn by puncturing the centrifuge tube with a needle.

Telomerase activity assay

One volume of extracts was mixed with one volume of 2× reaction buffer which contains: 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 β-mercaptoethanol, 20 μCi [α-^{32}P]dGTP (800 Ci/mmol NEN for a 40 μl total reaction volume) and 1–2 μM $(TTAGGG)_3$ oligonucleotide. The reaction was incubated at 30°C for 60 min and stopped with a solution containing 100 μg/ml RNase, 10 mM EDTA and 5 mM Tris–Cl, pH 7.5 at 37°C for 15 min. Elongation products were phenol-extracted, ethanol-precipitated in the presence of 1 μg/ml tRNA, run on an 8% acrylamide–7 M urea denaturing sequencing gel, and visualized by autoradiography as described (Counter *et al.*, 1992).

MPF activity assay

The assay for MPF activity was performed as described (Marshak *et al.*, 1991). In brief, the extracts were mixed with the substrate peptide in a buffer containing 50 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA and [γ-^{32}P]ATP, and incubated at 30°C for 12 min. The reaction was stopped by spotting on phosphocellulose paper (Whatman P81), washed four times in 100 mM phosphoric acid and dried, and the radioactivity was measured by scintillation counting.

Nuclear assembly assay

The reaction was carried out as described (Smythe and Newport, 1991). In brief, extracts were incubated with *Xenopus* sperm chromatin in the presence of 20 mM phosphocreatine, 2 mM ATP, pH 7.0, 5 mg/ml creatine kinase in 10 mM HEPES, pH 7.5 and 50% glycerol. Reactions were carried out at room temperature and aliquots were removed at different time points and diluted on a microscope slide with four volumes of DAPI solution. Morphology was followed by phase-contrast microscopy or fluorescence microscopy using a light microscope (Zeiss).

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