お茶の水女子大学自然科学報告　第49巻　第1号
Natural Science Report, Ochanomizu University, Vol. 49, No. 1(1998)

Examination of The Correlation between DNA-dependent Protein Kinase and Telomerase Activity

Fumi Ikeda[1], Yoshihisa Matsumoto[2], Norio Suzuki[2], and Kimiko Murakami-Murofushi[1]

[1]Department of Biology, Faculty of Sience, Ochanomizu University
[2]Department of Radiation Oncology, Faculty of Medicine, University of Tokyo

(Received June 19, 1998)

Summary

Telomerase is known as a ribonucleoprotein enzyme essential for the repair of replication-induced deletion of chromosome terminal in most eukaryotes. Although the regulatory mechanism of telomerase inactivation and re-activation has not yet been clarified, there is a possibility that telomerase activity is regulated by phosphorylation and dephosphorylation. DNA-dependent protein kinase (DNA-PK) is considered to be a critical enzyme in DNA double-strand break repair and possibility of involvement of this enzyme in the regulation of telomerase is expected. To examin correlation between telomerase and DNA-PK, we purified DNA-PK from human leukaemic MOLT-4 cells by successive column chromatography. Through the conventional process of purification, DNA-PK activity was accompanied by telomerase activity. We used modified purification protcol in this study, and finaly, succeeded in purification of DNA-PK separately from telomerase activity. This DNA-PK didn't show any effect on the telomerase activity, and these results indicate that the telomerase may not be regulated *via* phosphorylation with DNA-PK.

INTRODUCTION

The faithful replication of eukaryotic chromosome requires the activity of several DNA-dependent DNA polymerases, a DNA-dependent RNA polymerase (primase), and an RNA-dependent DNA polymerase that is the telomerase. Replication by DNA-dependent DNA polymerase alone does not completely duplicate the sequences at chromosome termini, resulting in a loss of telomeric sequence repeats with each cell division. Conditions that affect the balance in number of telomeric simple sequence repeats also affect chromosome stability, gene expression and likely additional cellular processes (1).

Telomerase, which is first detected in the cell free extracts of cilliates *Tetrahymena* by Greider and Blackburn (2), is a highly specialized telomere terminal transferase. This is an RNA-containing enzyme and adds telomeric repeats to the ends of chromosome using its own RNA as a template (3-6). Telomerase activity has been detected in most of mammalian tumor cells and germ cells (7-16). Telomerase activation was reported to be associated with cell transformation (17), and on the contrary differentiation of tumor cells is reported to be associated with pronounced down regulation of telomerase activity (13). But, recently, the mechanisms without innvolving telomerase have benn recognized to maintain telomere length by Bryan *et al.* (15). The presence of lengthened or stabilized telomeres is necessary for immortalization, and this may be achieved either by the reactivation of telomerase or by a novel and as yet unidentified mechanism(s).

Previously, we found a cell-cycle dependent telomerase activation using synchronized plasmodia of a true slime mold *Physarum polycephalum*(18). This observation suggests a possibility that phosphorylation and dephosphorylation may be a regulatory mechanism of telomerase activation and inactivation during cell cycle. Recently, He Li *et al.* indicated that protein phosphorylation reversibly regulated the function of telomerase and that protein phosphatase 2A (PP2A) might be one of telomerase inhibitory factors in the nucleus of human breast cancer cells(19).

DNA-dependent protein kinase (DNA-PK) is a nuclear serine/threonine kinase conposed of p470 catalytic subunit (DNA-PKcs) and p85/p70 heterodimer (Ku antigen) (20-24). DNA-PK binds to and requires DNA ends to be activated and phosphorylates a number of nuclear proteins including p53 (20,21) and replication protein A (25,26). DNA-PK is considered to be a critical enzyme in repairing double-starand breaks of DNA.

From these charactors of DNA-PK, a possibility that this emzyme may regulate the telomerase activity *via* phosphorylation/dephosphorylation is expected.

In this report, we purifeid DNA-PK separately from telomerase activity, and indicated that there is no correlation between telomerase activity and phosphorylating activity of DNA-PK.

MATERIALS and METHODS

Cells and culture. Molt-4 cells were grown at 37℃ in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) in suspention culture. Cells were harvested at 5-12×10^5cells per ml by centrifugation(1,000rp, 10min) and washed twice with ice-cold phosphate-buffered saline (PBS) and were stored at -80℃ until use.

DNA-PK purification. Molt-4 cell nuclei were prepared from 2-5×10^9 cells as described by Digmam *et al.* (27). And DNA-PK purification was performed essentially according to Matsumoto *et al.* (28) with some modification.

Cells were suspended in hypotonic buffer (10mM HEPES-NaOH, 1.5mM $MgCl_2$, 10mM KCl) followed by centrifugation at 1,800rpm for 10min. The pelet was nuclear fraction. Isolated nuclei were re-suspended in extraction buffer (20mM HEPES-NaOH, pH 7.9, 400mM KCl, 1mM EDTA, 1mM EGTA, 0.02% Tween 20, 10% glycerol, 1mM DTT, 1mM PMSF, 1μg/ml each of leupeptin, pepstatin and antipain) and agitated for 30 min followed by centrifugation at 100,000×g for 60min. The supernatant of nuclear extract (60ml) was passed through the first DEAE Bio-Gel A column and flow-through fraction was dialyzed against buffer A (20mM Tris-HCl, pH 7.5, 1mM EDTA, 10% glycerol, 50mM NaCl, 1mM DTT, 1mM PMSF, 1μg/ml each of leupeptin, pepstatin and antipain). Dialysate was applied to the second DEAE Bio-Gel A and eluted with buffer A with increasing NaCl concentration linearly from 0.05M to 0.5M. DNA-PK activity was eluted at 0.15-0.27M NaCl. The combined DNA-PK fraction was dialyzed against buffer B (20mM HEPES-NaOH, pH 7.2, 1mM $MgCl_2$, 15% glycerol, 200mM NaCl, 1mM DTT, 1mM PMSF, 1μg/ml each of leupeptin, pepstatin and antipain) and loaded to a native DNA-cellurose column(Pharmacia). Absorbed protein was eluted into 12 fractions (1ml each) by stepwise increase of NaCl concentratin in buffer B. Fraction 5 and 6, 0.6M NaCl elutes that contained DNA-PK activity, were dialyzed against buffer C containing 0.1M NaCl, and applied to Heparin Sepharose column. Absorbed proteins were eluted into 10 fractions (1ml each) by stepwise increase of NaCl concentration in buffer B. Fraction 3-6 (0.4-0.6M NaCl) were dialyzed against buffer B. Dialysate was finaly loaded second native DNA-cellurose column. Absorbed proteins were eluted in the same way, and fractions number 5 and 6 contained purifid DNA-PK.

Protein kinase assay. DNA-PK activity was assayed using α-casein as a substrate. The reaction mixture, final volume of 50μl, contained 20mM HEPES-NaOH, pH 7.2, 100mM NaCl, 5mM $MgCl_2$, 50μM [γ-^{32}P] ATP, 50μg of α-casein, 1mM DTT and 0.5mM each of NaF and β-sodium glycerophosphate, and 5μl of enzyme fraction was added. The reaction mixture was incubated at 37℃ for 10min and a 40μl aliquot was spotted onto a square 3MM paper (Whatman), washed with 5% trichloroacetic acid solution supplemented with 0.5% sodium pyrophosphate, and the radio activity retained was mesured with liquid sintilation counter.

Telomerase assay. Telomerase activity was assayed essentially according to Kim *et al.* (10). Briefly, enzyme fractions were incubated at 24℃ for 20min in 50μl reaction buffer containing 20mM Tris-HCl, pH 8.3, 0.1μg of telomerase substrate (5'-AATCCGTCGAGCAGAGTT-3'), 50μM each deoxynucleotide triphosphate, 1.5mM $MgCl_2$, 1mM EGTA, 63mM KCl, 0.005% Tween20, 1μg of T4 gene protein. To each reaction was then added 2 units of Taq DNA polymerase and 0.1μg of down stream primer CX (5'-CCCTTACCCTTACCCTTACCCTTA-3'), followed by 27-cycle polymerase chain reaction (94℃ for 30s, 50℃ for 30s, and 72℃ for 1min). five μl of the polymerase chain reaction mixture was then analyzed for *de novo* synthesized telomere by electrophoresis on 10% non-denaturing polyacrylamide gel followed by visualization with SYBER Green I and analyzed by FLA-2000 (FUJIFILM).

RESULTS and DISCUSSION

In order to know wheather telomerase activity is regulated by DNA-dependent protein kinase (DNA-PK), we tried to get purified DNA-PK and to examine the effect of DNA-PK upon the telomerase activity. With conventional purification procedures, telomerase activity was not separated with DNA-PK. Fig.1. shows a 2nd DEAE Bio-gel profile. Most of DNA-PK activity was eluted in fraction 16-27 (Fig.1A), though the telomerase activity was detected through all eluates (Fig.1B). In the1st native DNA-cellurose column chromatography, which is a final step of conventional DNA-PK purification, DNA-PK activity was eluted at 0.3 to 0.6M NaCl together with telomerase activity (deta not shown). After Heparin-Sepharose column chromatography, fraction containing DNA-PK activity were applied to 2nd native DNA-cellurose column. Fractions 5-6, the 0.6M NaCl eluate (Fig.2A lane 5-6) contained three major polypeptide bands of >170kDa, 85kDa and 70kDa, which were DNA-PK subunits. DNA-PK activity was eluted at 0.6M NaCl, fraction 5-6 (Fig.2B), and telomerase activity was eluted at 0.3M NaCl, fraction 2 (Fig.2C). Thus, DNA-PK was purified separately from telomerase activity.

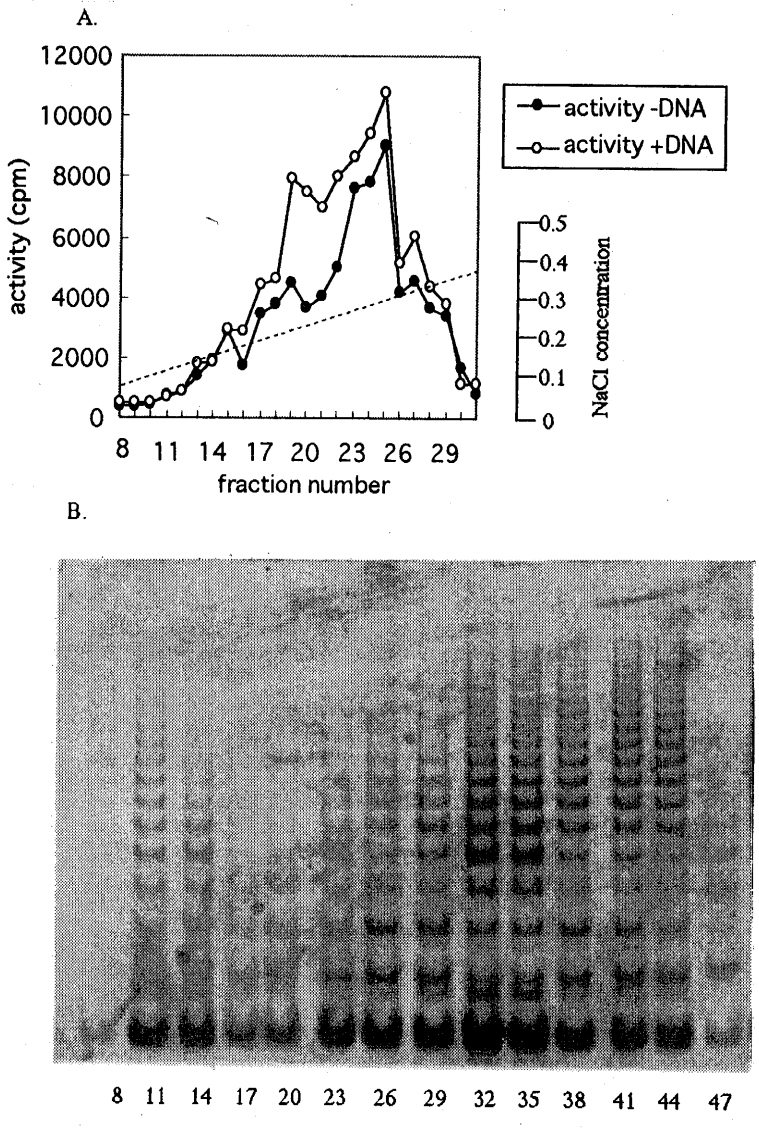

Fig.1. Elution profile of 2nd DEAE Bio-gel A column chromatography. (A) Protein kinase activity of 5-μl aliquot from each fraction was determined using α-casein as a substrate in the presense (open circle) or absence (closed circle) of 20ng/μl of sonicated calf thymus DNA at 37 ℃ for 10min. (B) Telomerase activity of a 2μl aliquot from each fraction was determined using TRAP assay (see MATERIALS and METHODS).

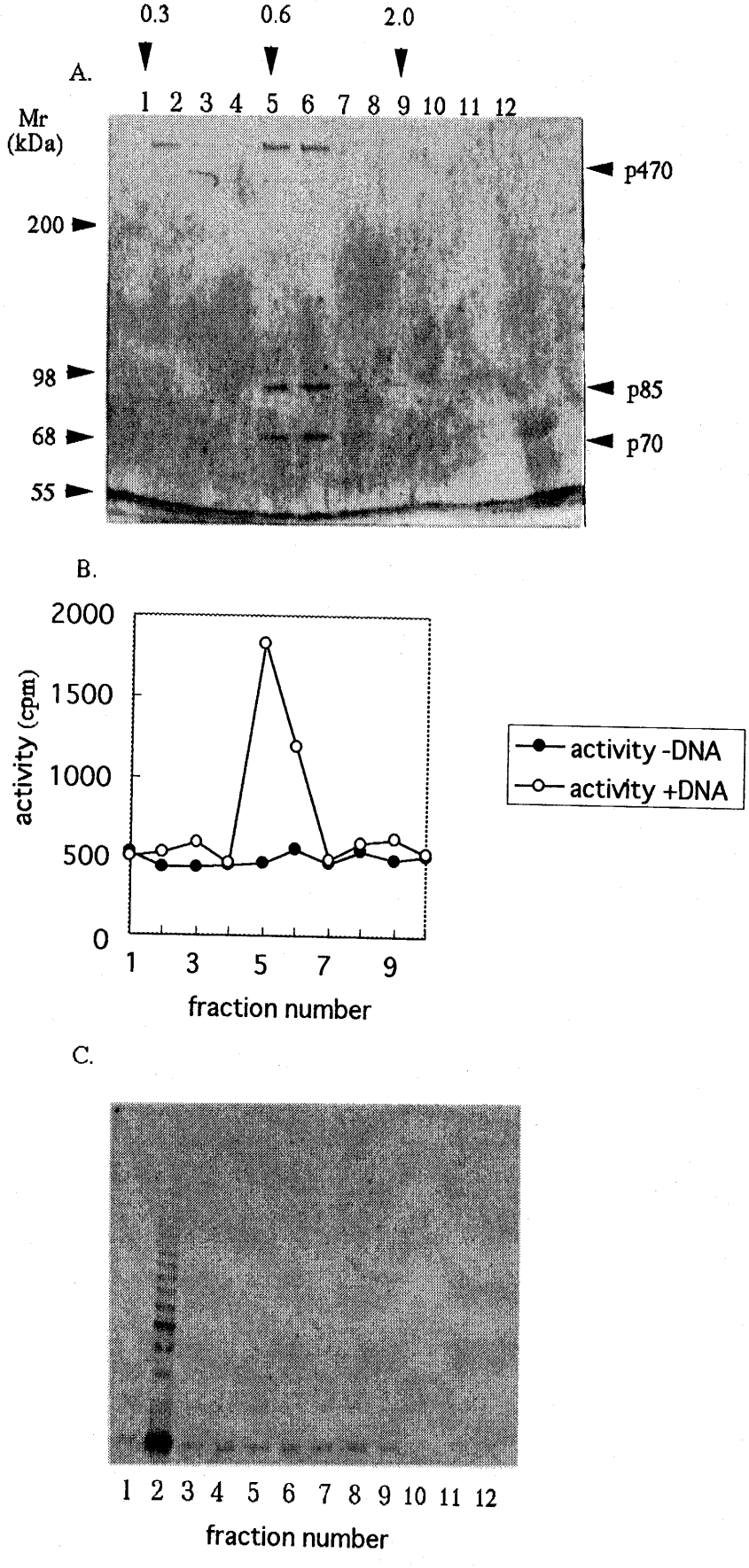

Fig.2. The elution profile of 2nd native DNA-cellulose column chromatography. (A) SDS-PAGE patterns of a 10μl aliquot from each fraction, visualized by Coomassie Brilliant Blue (CBB) staining. Fraction numbers are indicated at the top, and NaCl concentration is indicated with arrowheads. The position of p470, p85, and p70 are indicated by arrowheads. (B) Protein kinase activity of 5-μl aliquot from each fraction was determined using α-casein as a substrate in the presense (open circle) or absence (closed circle) of 20ng/μl of sonicated calf thymus DNA at 37℃ for 10min. (C) Telomerase activity of a 3μl aliquot from each fraction was determined using TRAP assay (see MATERIALS and METHODS).

Using the purified enzyme, we examined the effect of DNA-PK on telomerase activity under the phosphorylating conditions. But almost no effects were observed (deta not shown). DNA-PK might not affect the regulation of telomerase activity.

To know the regulatory mechanisms of telomerase activity in the cell cycle, we are now studying the involvement of some protein kinases other than DNA-PK. But very recently, a report was published that Ku antigen might have some biological function at telomere region of chromosome (29), and the posibility of the involvement of DNA-PK on telomerase reguration in a different way is still left. Our purification method might be very useful to clarify this problem and other problems that DNA-PK related to.

REFERENCES

1. Blackburn, E. H. (1994). Telomeres: no end in sight. Cell 77, 621-623.
2. Greider, C. W., and Blackburm, E. H. (1985) Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. Cell **43**, 405-413.
3. Greider, C. W., and Blackburm, E. H. (1987) The telomere terminal transferase of *Tetrahymena* is a ribonucleoprotein enzyme with two kinds of primer specificity. Cell **51**, 887-898.
4. Greider, C. W., and Blackburm, E. H. (1989) A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis. Nature **337**, 331-337.
5. Shippen-Lentz, D., and Bluckburn, E. H. (1990) Functional evidence for an RNA template in telomerase. Science **247**, 546-552.
6. Blackburn, E. H. (1992) Telomerases. Annu. Rev. Biochem. **61**, 113-129.
7. Morrin, G. B. (1989) The human telomere terminal transferase enzyme is a ribonucleoorotein that synhtesizes TTAGGG repeats. Cell **59**, 521-529.
8. Counter, C. M., Avilion, A. A., LeFeuvre, C. E., Stewart, N. G., Greider, C. W., Harley, C. B., and Bacchetti, S. (1992) Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. EMBO J. **11**, 1921-1929.
9. Counter, C. M., Hirte, H. W., Baccheti, S., and Harley, C. (1994) Telomerase activity in human ovarian carcinoma. Proc. Natl. Acad. Sci. USA **91**, 2900-2904.
10. Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L., Coviello, G. M., Wright, W. E., Weinrich, S. L., and Shay, J. W. (1994) Specific association of human telomerase activity with immortal cells and cancer. Science **266**, 2011-2015.
11. Broccoli, D., Young, J. W., and Lange, T. (1995) Telomerase activity in normal and malignant hematopoietic cells. Proc. Natl. Acad. Sci. USA **92**, 9082-9088.
12. Tahara, H., Nakanishi, T., Kitamoto, M., Nakashiro, R., Shay, J. W., Tahara, E., Kijiyama, G., and Ide, T. (1995) Telomerase activity in human liver tissues: comparison between chronic liver disease and hepatocellular carcinomas. Cancer Res. **55**, 2734-2736.
13. Sharma, H. W., Sokoloski, J. A., Pres, J. R., Maltese, J. Y., Sartorelli, A. C., Nichols, C. G., Khaled, Z., Telang, N. T., and Narayanan, R. (1995) Differentiation of immortal cells inhibits telomerase activity. Proc. Natl. Acad. Sci. USA **92**, 12343-12346.
14. Counter, C. M., Gupta, J., Harley, C.B., Leber, B., and Bacchetti, S. (1995) Telomerase activity in normal leukocytes and in hematologic malignancies. Blood **85**, 2315- 2320.
15. Bryan, T. M., Englezou, A., Gupta, J., Bacchetti, S., and Reddel, R. R. (1995) Telomere elongation in immortal human cells without detectable telomerase activity. EMBO J. **14**, 4240-4248.
16. Hiyama, E., Hirayama, K., Yokoyama, T., Matuura, Y., Piatyszek, M. A., and Shay, J. W. (1995) Correlating telomerase activity levels with human neuroblastoma outcomes. Nat. Med. **1**, 249-255.
17. Counter, C. M., Botelho, F. M., Wang, P., Harley, C. B., and Bacchetti, S. (1994) Stabilization of shotr telomeres and telomerase activity accompany immortalization of Epstein-Barr virus-transformed human B lymphocytes. J. Virol. **68**, 3410-3414.
18. Shimada, Y., Nakano, M., Kanda, N., Murofushi, K. M., Kim, J. K., Ide, T., Murofushi, H. (1997) Cell cycle-dependent activation of telomerase in naturally synchronized culture of true slime mold, *Physarum polycephalum*. Biochnem. Biophys. Res. Commun. **232**, 492-496.
19. Li, H., Zhao, L. -L., Funder, J. W., and Liu, J. -P. (1997) Protein phosphatase 2A inhibits nuclear

telomerase activity in human breast cancer. J. Biol. Chem. **272**, 16729-13732.
20. Lees-Miller, S. P., Chen, Y. -R., and Anderson, C. W. (1990) Human cells contain a DNA-activated protein kinase that phosphorylates simian virus 40 T antigen, mouse p53, and the human Ku autoantigen. Mol. Cell. Biol. **10**, 6472-6481.
21. Carter, T., Vancurova, I., Sun, I., Lou, W., and Deleon, S. (1990) A DNA-activated protein kinase from HeLa cell nuclei. Mol. Cell. Biol. **10**, 6460-6471.
22. Dvir, A., Stein, L. Y., Calore, B. L., and Dynan, W. S. (1993) Purification and characterization of a template-associated protein kinase that phosphorylates RNA polymerase II. J. Biol. Chem. 268, 10440-10447.
23. Gottlieb, T. N., and Jackson, S. P. (1993) The DNA-dependent protein kinase: requirement for DNA ends and association with Ku antigen. Cell **72**, 131-142.
24. Hartley, K. O., Gell, D., Smith, G. C. M., et al. (1995) DNA-dependent protein kinase catalytic subunit: a relative of phosphatidylinositol 3-kinase and the *ataxia telangiectasia* gene product. Cell **82**, 849-856.
25. Pan, Z. -Q., Amin, A. A., Gibbs, E., et al. (1994) Phosphorylation of the p34 subunit of human single-stranded-DNA-binding protein in cyclin A-activated G1 extracts is catalyzed by cdk-cyclin A complex and DNA-dependent protein kinase. Proc. Natl. Acad. Sci. USA **91**, 8343-8347.
26. Brush, G. S., Anderson, C. W., and Kelly, T. J. (1994) The DNA-activated protein kinase is required for the phosphorylation of replication protein A during simian virus 40 DNA replication. Proc. Natl. Acad. Sci. USA **91**, 12520-12524.
27. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nuc. Acids. Res.**11**, 1475-1489.
28. Matsumoto, Y., Suzuki, N., Sakai, K., Morimatsu, A., Hirano, K., and Murofushi, H. (1997) A possible mechanism for hyperthermic radiosensitization medeated through hyperthermic lability of Ku subunits in DNA-dependent protein kimase. Biochnem. Biophys. Res. Commun. **234**, 598-572.
29. Gravel, S., Larrivee, M., Labrecque, P., Wellinger, R. J. (1998) Yeast Ku as a regulator of chromosomal DNA structure. Science **280**, 741-744.